THE MOLECULAR ACTION OF CHOLERA TOXIN IN RABBIT INTESTINAL EPITHELIAL CELLS

DAVID LONGBOTTOM

Ph.D.

UNIVERSITY OF EDINBURGH

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To my Mum and Dad

PREFACE

The work reported in this thesis was carried out between 1st October 1985 and 30th September 1988 under the supervision of Dr. Simon van Heyningen at the Department of Biochemistry, University of Edinburgh Medical School, in Edinburgh. All material presented in this thesis, unless otherwise stated, is the sole work of the author, as is the composition.

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ABBREVIATIONS

A_1	Active peptide of cholera toxin subunit A
AC	Adenylate cyclase
ADP	Adenosine 5/-diphosphate
ADPR	Adenosine diphosphoribose
ATP	Adenosine 5/-triphosphate
GC	Guanylate cyclase
BSA	Bovine serum albumin
CaM	Calmodulin
Cyclic AMP, cAMP	Adenosine 3/,5/-cyclic monophosphate
Cyclic GMP, cGMP	Guanosine 3/,5/-cyclic monophosphate
Da	Dalton
DAG	Diacylglycerol
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
G_i , N_i	The inhibitory regulatory subunit of adenylate cyclase
G_s , N_s	The stimulatory regulatory subunit of adenylate cyclase
G_{M1}	Galactosyl-N-acetylgalactosaminyl [N-acetylneuraminyl]-galactosylglucosyl ceramide
GTP	Guanosine 5/-triphosphate
HEPES	N-2-Hydroxyethylpiperazine-N/-2-ethane-sulphonic acid
5-HT	5-Hydroxytryptamine
IBMX	3-Isobutyl-1-methylxanthine
INT	2-[4-Iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride
IP_3	Inositol 1,4,5-trisphosphate
Isoniazid	Isonicotinic acid hydrazide
A-kinase	cAMP-dependent protein kinase

C-kinase	Protein kinase C
G-kinase	cGMP-dependent protein kinase
min	Minute
MOPS	3-[N-Morpholino]propanesulphonic acid
NAD^+	β-Nicotinamide adenine dinucleotide
NADase	NAD^+-glycohydrolase
NADPH	β-Nicotinamide adenine dinucleotide phosphate, reduced form
PAGE	Polyacrylamide gel electrophoresis
PGE_2	Prostaglandin E_2
PI-4,5P_2	Phosphatidyl inositol 4,5-bisphosphate
PL-C	Phospholipase C
s	Second
SDS	Sodium dodecyl sulphate
TRIS	Tris[hydroxymethyl]aminomethane
v/v	Volume/volume
w/v	Weight/volume

ABSTRACT

The mechanisms by which cholera toxin causes its characteristic diarrhoea were studied in rabbit intestinal epithelial cells, in contrast to most of the previous work on the toxin which was carried out on cell types not involved with the pathology of the disease. Brush border and basal-lateral membranes were prepared from intestinal epithelial cells by differential centrifugation and $MgCl_2$ precipitation. The purity of the membrane fractions was assessed by marker enzyme analysis; sucrase activity was 18.4-fold greater in brush border than in basal-lateral membranes, whereas (Na^+-K^+)ATPase activity was only 2.8-fold greater in basal-lateral than in brush border membranes. The lower enrichment factor for (Na^+-K^+)ATPase is due to the heterogeneous distribution of basal-lateral membranes which occurs when the technique of $MgCl_2$ precipitation is used. The active A_1 peptide of cholera toxin catalyzed the transfer of the ^{32}P-ADP-ribose moiety of [adenylate-^{32}P]NAD^+ to three proteins of molecular mass 45, 40 and 37 kDa in brush border membranes only. This ADP-ribosylation caused a 20% release of the 45 and 40 kDa proteins from the membranes after an incubation time of 20 min. The 40 kDa protein has the same molecular mass as the α subunit of the stimulatory regulatory component ($G_{s\alpha}$) of adenylate cyclase. Both cholera toxin and sodium fluoride activated the catalytic component of adenylate cyclase in basal-lateral membranes only, but by first modifying the same regulatory component in brush border membranes. Thus, in the intact cell, $G_{s\alpha}$ is released from the brush border membrane into the cell

cytosol upon ADP-ribosylation by cholera toxin and moves to the other side of the cell, i.e. the basal-lateral membrane, where it activates adenylate cyclase. The resulting elevation in the cyclic AMP content of the cell (induced by cholera toxin) activated cyclic-AMP-dependent protein kinases which increased the phosphorylation of two proteins of 53 and 42 kDa, and caused the unique phosphorylation of a 36 kDa protein and possibly two of 45 and 30 kDa, all in the brush border membrane. These changes in the phosphorylation of proteins in the presence of cholera toxin are thought to be responsible for controlling the decrease in Na^+-Cl^- absorption in villus cells and increase in Cl^- secretion in crypt cells, resulting in a massive water loss. The effects of six antipsychotic drugs (phenothiazines) on the ADP-ribosylation of $G_{s\alpha}$ and the activation of adenylate cyclase was investigated. A correlation was found between the effects of antidiarrhoeal drugs on the ADP-ribosylation of $G_{s\alpha}$ and the activation of adenylate cyclase with their reported antidiarrhoeal activities and abilities to bind calmodulin: the greater the drugs' affinity for calmodulin and ability to inhibit the diarrhoea, the greater was the inhibition of ADP-ribosylation and adenylate cyclase. Since calmodulin is involved in the activation of many different enzymes and the drugs bind calmodulin with varying affinities, it is possible that the drugs inhibit the diarrhoea by binding to calmodulin and so inhibiting ADP-ribosylation and adenylate cyclase activity.

CHAPTER ONE

INTRODUCTION

1.1 Cholera the disease

Cholera is a disease that has plagued the world throughout history, assuming pandemic (meaning spread rapidly over a wide geographical area) proportions on seven occasions since 1817 (Garfield, 1986). It is a massive diarrhoea resulting from an enormous secretion of fluid (over a litre per hour) into the small intestine, leading to dehydration, kidney failure, circulatory collapse and ultimately death in about 50% of cases if left untreated. Although it is not the most prevalent of the diarrhoeal diseases it certainly causes the most severe fluid loss, and is therefore responsible for a large proportion of life-threatening illness and death during the cholera season in endemic areas. It is estimated that each year there are approximately 1,000 million cases of acute diarrhoea in children under 5 years of age in Asia, Africa and Latin America, resulting in 5 million deaths (Holmgren, 1981).

Despite all the major advances that we have seen in medicine and public health, the increase in volume of international traffic has provided a good means for spreading cholera beyond the endemic areas.

1.1.1 History

The first recorded cholera pandemic began in 1817 in Jessore (now Bangladesh), India (Longmate, 1966). The term 'Asiatic Cholera' was used to distinguish this disease from other similar illnesses. This Asiatic Cholera swept through India, across the Arabian Sea to the Middle East and into

Russia. In 1832 almost every European capital had been affected. This type of worldwide cholera epidemic would recur six more times.

The current, seventh pandemic began back in 1961 in Indonesia, from where it spread to Southeast Asia, the mainland of Asia and through the Middle East, reaching Africa in 1970. By 1973 cholera was endemic in Malawi, Mozambique and Angola. The disease is now apparently endemic along the United States Gulf Coast.

The cause of the disease is the comma-shaped gram-negative bacterium *Vibrio cholerae* which was first identified and isolated by Pacini in 1854, and later reaffirmed by Robert Koch in 1883. Koch suggested that the characteristic diarrhoea of cholera was due to a poison produced by the bacteria in the small intestine. His reason for believing this was that the invading bacteria were only found in the contents of the patient's intestine and not in the surrounding tissues or the epithelial wall. However, this idea of cholera being a toxicosis was not substantiated because intraperitoneal injections of the culture filtrate of *V. cholerae* failed to produce symptoms of the disease in experimental animals.

It was this difficulty in finding suitable animal models that was apparently the main reason for the long lapse in studies on the pathogenesis of cholera. Oral administration of even large amounts of live *V. cholerae* either failed to

induce the infection or killed the animal through septicaemia rather than diarrhoea. Also the findings that the intestinal epithelium of cholera victims was desquamated and sloughed further led to the misconception of the aetiology of cholera. Thus, the disease state of cholera was generally thought to arise from denudation of the intestinal surface by the invading bacteria, possibly through the action of digestive enzymes and mucinase (Burnet and Stone, 1947), resulting in the exudation and loss of body fluid. This view prevailed through the first half of the 20th century although great physicians of the mid-19th century had noted, even before the discovery of *V. cholerae*, that the characteristic rice-water stool in cholera consisted of water and "blood salts" but contained very little albuminous matter, suggesting a role of poison rather than physical lesion in the disease (Finkelstein, 1973).

It was not until the great Egyptian epidemic in 1947 and the Thai epidemic in 1958 that better observations and procedures were made on the composition and dynamics of electrolytes and fluid movements. This was to form the basis of fluid replacement therapy which was to save many millions of lives from cholera (see section 1.1.3). The rapid recovery of patients seemed to indicate again that poisoning was the cause of the illness. In the late 1950's there was to be a resurgence of interest in cholera due to these observations.

In 1953 De and Chatterje developed the first successful animal model for cholera. They managed to collect "rice-water" which had accumulated in ligated segments of the small intestine of rabbits after they had been injected with a live *vibrio* suspension.

In 1959 De and Dutta *et al.* demonstrated that the bacterium secretes enterotoxin, a diarrhoea-causing agent, by producing experimental cholera in the animal model with a cell-free culture filtrate of *V. cholerae*. By this time the technique for biopsy of the small intestine was available and in 1960 Gangarosa *et al.* reported that the small intestine of cholera patients showed no evidence of damage during profuse diarrhoea. Thus, the desquamation of the intestine observed earlier was now known to be due to a post-mortem artefact and not due to the disease.

The toxin was first isolated in 1968 by Richardson and Evans and then by Finkelstein and LoSpalluto (who called it choleragen) in 1969. The toxin was found to consist of several subunits. During the final step of the purification procedure a protein which cross-reacted with anti-cholera toxin antibody but was not enterotoxic was separated from the enterotoxin and was named choleragenoid. Choleragen and choleragenoid were obtained in crystalline form in 1972 (Finkelstein and LoSpalluto).

Field (1971), using the purified material, showed that cholera toxin greatly stimulated the flow of electrolytes, particularly Na^+ and Cl^-, across the ileal mucosa, from the serosa to the lumen. This event was mimicked by cyclic AMP or substances such as theophylline that increased the cyclic AMP content of tissues (reviewed by Field, 1980). It was proposed that cholera toxin elevated the cyclic AMP level in enterocytes and then some cyclic-AMP-dependent biochemical process changed the flow of electrolytes through the intestinal epithelium. This hypothesis was soon supported by the observation that the cyclic AMP content in the mucosa was increased by placing cholera toxin in the intestinal lumen (Schafer *et al.*, 1970). Cholera toxin was later shown to activate adenylate cyclase in the small intestine without affecting the cyclic nucleotide phosphodiesterase activity (Kimberg *et al.*, 1971; Sharp and Hynie, 1971), in other words the activation of the cyclase was not due to inhibition of the breakdown of cyclic AMP. Furthermore, cholera toxin could induce lipolysis in fat cells, which was mediated by cyclic AMP (Vaughan *et al.*, 1970). This and other observations suggested that the activation of adenylate cyclase was the primary action of cholera toxin not only in the intestine but also in other cells and tissues. It is now clear that cholera toxin can activate adenylate cyclase in almost all vertebrate cells.

Two strains of *V. cholerae* have been identified, the common form, which is the one that was discovered by Pacini, and the classical form, which was isolated in 1905 and is called

V. cholerae el tor. The common form is thought to have been the major cause of epidemic cholera for the first six pandemics whereas *V. cholerae el tor* is the primary cause of the present seventh pandemic, virtually replacing *V. cholerae* as the main epidemic strain. However, it has been noted by the International Centre for Diarrhoeal Disease Research in Dacca, Bangladesh that as recently as 1982 *V. cholerae* is again gaining advantage as the major cause of cholera (Samadi *et al.*, 1983). *V. cholerae el tor* can survive much better in the environment than *V. cholerae* and causes a broader range of disease (Mackay, 1980). *V. cholerae* immobilizes patients reducing their capacity for spreading the disease while *V. cholerae el tor* produces moderate cases allowing victims to move around with only mild discomfort. This means that these victims act as dangerous vectors of the disease.

1.1.2 Transmission

The mode of transmission of cholera has been the subject of debate since the early 1800's. In 1849 it was discovered by John Snow that cholera is a waterborne infection and he suggested that it is a contagious disease caused by a poison reproducing itself in the bodies of its victims and spreading through excretions and vomit that then contaminate the water supply. Cholera can be transmitted by eating contaminated food as well as by drinking contaminated water. In 1978 a cholera outbreak along the Gulf Coast of Louisiana turned out to be due to cooked crabs from the Louisiana marshes (Blake *et al.*, 1980).

Thus, *V. cholerae* is spread by food and water which have been contaminated by human excrement. The bacteria thrive in the human digestive tract where they multiply and produce the enterotoxin. This toxin binds to the intestinal lining, triggering the hypersecretion of fluids into the lumen of the intestine leading to the characteristic watery diarrhoea of this disease. As much as 25% of the body's fluids are drained within hours as well as depleted of essential salts. The result is that the patient faces dehydration and metabolic acidosis, leading to a painful death within a few hours unless treatment is administered (Mackay, 1980).

1.1.3 Treatment and control

Cholera patients suffer so much fluid loss that unless they are treated quickly they die from dehydration long before their bodies have time to mount an immune response to the infecting bacteria. The treatment is very simple, consisting of the replacement of lost fluids and the infusion of salts to restore the body's water and chemical balance. This is almost always effective but 58% of victims die if they do not receive prompt medical attention.

In Third World countries, where cholera is most prevalent, any kind of treatment must be simple, inexpensive and available to large numbers of patients. The easiest method is that of oral rehydration therapy in which essential fluids and salts that are lost during the severe diarrhoea and vomiting are replaced. This type of therapy can reduce the mortality to less than 1%.

As far as antibiotics are concerned there has not been much success in the treatment of cholera. However, if used in conjunction with oral rehydration, drugs reduce the volume of fluid loss and hence the amount of replacement fluid necessary.

Epidemiological observations have also indicated the development of an acquired immunity by infected individuals. In fact, in endemic areas the incidence of cholera infection has been found to be higher in children under 4 years of age, while it is diminished in older people. This has been found to be due to an increase in the cholera antibody with age (Mosley *et al.*, 1968).

Although a century of research has passed, there has not been a satisfactory vaccine developed. At first, research involved trying to develop a vaccine that would stimulate the immune system to fight the invading bacteria. However, antibacterial cholera vaccines have an efficiency of only 40-80% for a short duration of 3-6 months (Feeley and Gangarosa, 1980). Since De's discovery of the enterotoxin, research has been redirected to find a vaccine that will spark the immune system in the gut to fight the toxin rather than the bacteria.

Another approach to vaccination has been to construct a weakened or attenuated strain by recombinant DNA techniques (Kaper *et al.*, 1984). The genes encoding toxin production are removed, and the genes encoding the antigens likely to

be involved in immunity are preserved. This strain can then be administered as an oral vaccine, stimulating antibody production to protect the body from future bacterial invasion while not producing the severe symptoms associated with the disease.

As well as using vaccines and drugs though, health education is a major factor in lowering the incidence of cholera. Azurin and Alvero (1974) of the joint Philippines/Japan/WHO Cholera El Tor Research Project, found that sanitation measures in conjunction with an uncontaminated water supply reduced the number of cholera cases by 76%.

1.2 Structure and function of cholera toxin

1.2.1 Structure of cholera toxin

Cholera toxin has been shown, by sedimentation equilibrium ultracentrifugation, to have a molecular mass of approximately 82-86 kDa (LoSpalluto and Finkelstein, 1972; Sattler *et al.*, 1975; van Heyningen, 1976; Lai *et al.*, 1976). This protein, which can be easily purified from culture filtrates of *V. cholerae* (Finkelstein and LoSpalluto, 1969), contains no detectable amounts of carbohydrate or lipid (LoSpalluto and Finkelstein, 1972). Crystals of the toxin have also been obtained (Finkelstein and LoSpalluto, 1972). The molecule has been found to be very stable in that it can refold to an active configuration after being treated with SDS or 6M guanidinium chloride.

Analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) has shown the toxin to consist of two types of subunits, called A and B, which are non-covalently associated (Finkelstein *et al.*, 1972; Cuatrecasas *et al.*, 1973; van Heyningen, 1974). Further studies revealed that there is one A subunit (27-30 kDa) and several identical B subunits (8-14 kDa) (van Heyningen, 1974; Finkelstein *et al.*, 1974; Gill, 1976a; Kurosky *et al.*, 1977). The B subunits exist as an aggregate even in the presence of SDS due to strong non-covalent interactions between them, and can only be separated by boiling in SDS. The bacterium synthesizes subunit A as a single precursor polypeptide chain which is rapidly nicked into two chains by bacterial proteases (Gill and Rappaport, 1979; Mekalanos *et al.*, 1979b). These two chains, called A_1 (18-25 kDa) and A_2 (5-9.7 kDa), are covalently linked by a disulphide bond. The nicking of the A subunit does not produce an appreciable decrease in the molecular mass, suggesting that few amino acids, if any, are lost. The precursor polypeptide has a NH_2-terminal sequence similar to that of the A_1 fragment and the same COOH-terminal amino acid (Leu) as the A_2 fragment (Duffy *et al.*, 1981a). Thus, the precursor has the sequence $NH_2-A_1-A_2-COOH$.

1.2.2 Subunit arrangement

From calculations of molar ratios using the molecular masses of each subunit and of the whole toxin, Lai (1977) proposed that there are five B subunits for every A subunit. This ratio has been verified by Gill (1977), van Heyningen

(1977b) and Ribi *et al.* (1988). The only way of arranging these subunits whilst maintaining a symmetrical structure is for the B subunits to be in a ring with the A subunit on the axis (Fig. 1-1) (Gill, 1976a; van Heyningen, 1977a; Ribi *et al.*, 1988). Evidence supporting this model comes from high resolution electron microscopy which shows molecules in a ring form (Ohtomo *et al.*, 1976) and from cross-linking experiments with bifunctional reagents (Gill, 1976a). X-ray diffraction analysis of the crystalline toxin is also consistent with five B subunits per molecule (Sigler *et al.*, 1977; Ribi *et al.*, 1988).

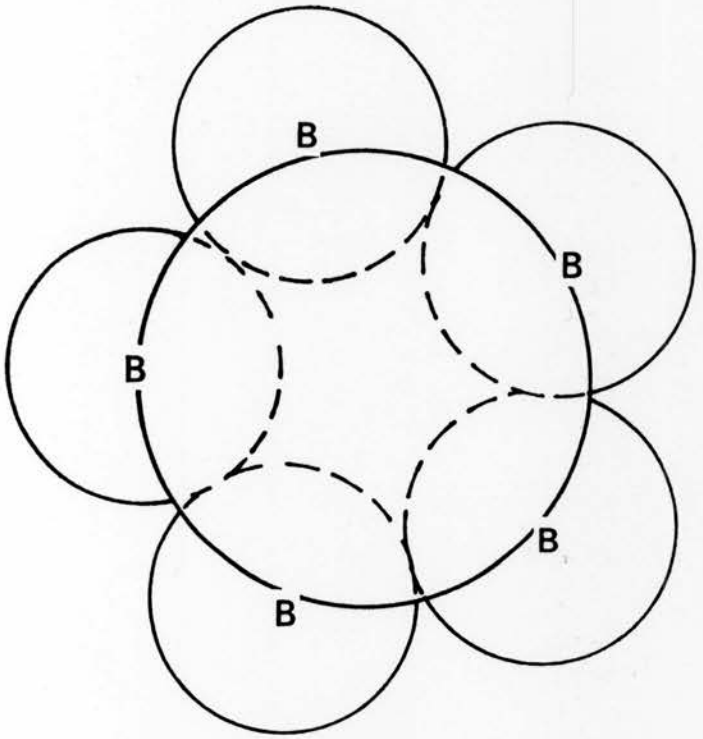
Mekalanos *et al.* (1979b) and Tomasi *et al.* (1979) have shown that the A subunit appears to be associated with the B subunits via the A_2 fragment. They found that the structure of the toxin was not altered by reducing the disulphide bond between the A_1 and A_2 fragments and that there was no dissociation of the A_1 or A_2 from the B subunits. However, in the presence of SDS A_1 was released leaving the A_2-5B complex. Thus, it seems apparent that as well as the disulphide bond, non-covalent bonds are important in the binding of the A_1 fragment to the A_2-5B complex.

1.2.3 Choleragenoid

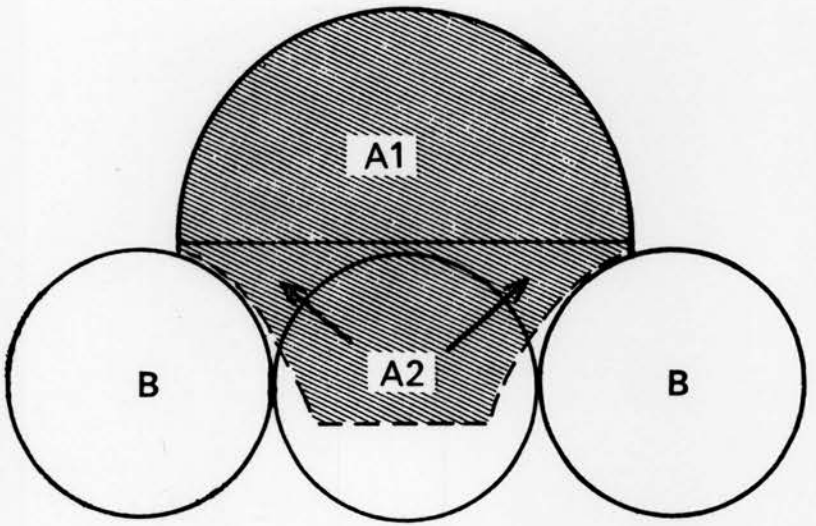
As well as secreting the toxin the bacterium secretes an aggregate of the five B subunits, without the A subunit, which immunologically cross-reacts with cholera toxin but has no toxic activity. This protein is called choleragenoid and it was shown to be the same as the pentamer by

Fig. 1-1 **Proposed model for the structure of the cholera toxin molecule.**

Taken from van Heyningen (1977a).



Plan



Elevation

determining its molecular weight by sedimentation equilibrium ultracentrifugation. The molecule was found to have a molecular mass of 54.3-58 kDa (LoSpalluto and Finkelstein, 1972; van Heyningen, 1976) which is identical to the molecular mass of the B subunit aggregate (Sattler *et al.*, 1975; Lai *et al.*, 1976). Further evidence was revealed by SDS-PAGE where only the B subunit was observed in choleragenoid (Finkelstein *et al.*, 1972; Cuatrecasas *et al.*, 1973; Lönnroth and Holmgren, 1973; van Heyningen, 1974). A mutant of *V. cholerae* called Texas Star, which only produces the B subunits, has been isolated by Honda and Finkelstein (1979).

1.2.4 The primary structure and structural genes of the subunits

The complete amino acid sequence of both subunits is known (Nakashima *et al.*, 1976; Lai, 1977; Lai *et al.*, 1979; Duffy *et al.*, 1981b; Mekalanos *et al.*, 1983). The B subunit is composed of 103 amino acid residues (see Appendix A), has a molecular mass of 11,604 Da, which is consistent with the subunit composition of AB_5 for the holotoxin (Lai, 1977), and shows similarity to the B chains of glycoprotein hormones such as thyrotropin, luteinizing hormone and follicle stimulating hormone (Ledley *et al.*, 1976; Kurosky *et al.*, 1977). The A subunit is composed of 240 amino acid residues (see Appendix B), of which 193 (or 192) make up the A_1 peptide (Lai *et al.*, 1979; Mekalanos *et al.*, 1983) and 46 (or 47) make up the A_2 peptide (Duffy *et al.*, 1981b; Mekalanos *et al.*, 1983): the reason for this discrepancy is

that sometimes the two peptides are cleaved between Ser 193 and Ser 194, and sometimes they are cleaved between Arg 192 and Ser 193 as well as between Ser 194 and Met 195 with the loss of the two serine residues (see Appendix B).

The structural genes, ctxA and ctxB, of the toxin have been cloned and their complete nucleotide sequence determined (Pearson and Mekalanos, 1982; Mekalanos *et al.*, 1983). The two genes are cotranscribed from a polycistronic operon (Mekalanos *et al.*, 1983) which is part of a 7-12 kilobase genetic element (Mekalanos, 1983), with the A cistron (ctxA) preceding the B cistron (ctxB) (Pearson and Mekalanos, 1982). The nucleotide sequence of the ctx gene suggests that both ctxA and ctxB are cotranscribed from a single promoter, which precedes the ctxA gene (Rosenberg and Court, 1979).

Several genetic factors influence the level of cholera toxin production. The first is the copy number of the toxin genetic element. Amplification of this element increases the production of the toxin (Mekalanos, 1983). Secondly, the number of tandem repeats of the sequence TTTTGAT upstream of the ctx promoter affects toxin expression (Miller and Mekalanos, 1984), and thirdly, there are at least two regulatory genes, one whose product stimulates the transcription of ctxAB and one which may encode a negative regulatory factor (Miller and Mekalanos, 1984).

1.2.5 Role of each subunit

Both the A and B subunits are required for the expression of toxicity (Finkelstein *et al.*, 1974; Sattler *et al.*, 1975; Gill, 1976a). The B subunit has been shown to bind to ganglioside G_{M1}, a cell-surface glycolipid, but this binding is not toxic to cells. Subunit A does not bind ganglioside significantly but the A_1 peptide alone can activate adenylate cyclase in cell-free extracts. Therefore, the A_1 fragment must be transported to the cytoplasmic side of the cell's plasma membrane where it activates adenylate cyclase by the ADP-ribosylation of the regulatory component of adenylate cyclase. The details of these events will be described in the following sections.

1.3 Interaction of cholera toxin with cell membranes

1.3.1 Ganglioside G_{M1} as the receptor

The initial event of the action of the toxin is to bind to the cell membrane via some specific receptor. It was suggested by van Heyningen *et al.* (1971) that gangliosides are the receptors for cholera toxin. This was deduced from their observation that the preincubation of cholera toxin with mixed gangliosides neutralized the toxic activity. Subsequently, ganglioside G_{M1} was shown to have the strongest inhibitory effect, being 50-100 times greater than G_{D1a}, G_{M2}, G_{M3} etc (Cuatrecasas, 1973a; King and van Heyningen, 1973). The neutralization was explained by the binding of the toxin to the free G_{M1} thus preventing it from binding to cell surface receptors and producing its effects.

Other evidence agreeing with ganglioside G_{M1} being the toxin receptor came from the work of Cuatrecasas (1973b) who showed that there is a 10-fold increase in the response of fat cells to toxin after preincubation of cells with ganglioside. Pigeon erythrocytes were also made more responsive to toxin by preincubation with ganglioside (Gill and King, 1975; King *et al.*, 1976). The best evidence came from using cells in culture whose ganglioside content could be measured and altered artificially. Hollenberg *et al.* (1974) looked at toxin binding in three different mouse cell lines transformed with viruses and found a correlation between the ganglioside G_{M1} content, toxin binding and the activation of adenylate cyclase.

1.3.2 Interaction of the B subunit with ganglioside G_{M1}

The first indications that the B subunits were responsible for the binding of the toxin to ganglioside G_{M1} came from observations that choleragenoid protects cells and intact loops of small intestine (Pierce, 1973) from the action of the toxin (Cuatrecasas, 1973a; van Heyningen, 1974; Holmgren *et al.*, 1974; Gill and King, 1975). This is probably due to the ganglioside binding sites being occupied by subunit B and so not available for reaction with the toxin.

Cholera toxin was found to bind 4 (Sattler *et al.*, 1978) or 5-6 (Fishman *et al.*, 1978) molecules of the oligosaccharide portion of ganglioside G_{M1}, indicating that each B subunit has one binding site for the ganglioside. The terminal galactose residue of ganglioside G_{M1} was shown to be

involved in the binding of cholera toxin by Mullin *et al.* (1976) and is protected by toxin from modification by galactose oxidase and subsequent reduction with ^{3}H-labelled sodium borohydride (Mullin *et al.*, 1976). Subunit A does not bind ganglioside, at least no more so than most other proteins and gangliosides, presumably because of hydrophobic interactions.

1.3.3 Entry of the toxin into the cell

The active A_1 peptide of the toxin must penetrate the cell membrane in order to gain access to the adenylate cyclase which is located on the cytoplasmic surface of the membrane. Unfortunately, the mechanism by which this occurs is still not known.

A lag phase of 15-90 minutes, depending on the cell type and conditions of incubation, has been observed between the binding of the toxin to the cell and the increase in activity of adenylate cyclase (Cuatrecasas, 1973c; Bennet and Cuatrecasas, 1975; Gill and King, 1975; Fishman, 1980). This was suggested to be due to the time taken for the A_1 peptide to dissociate from the toxin molecule and enter the cell. Confirmation came in 1975 (van Heyningen and King; Gill and King) when there was shown to be no lag in lysed pigeon erythrocytes, where the toxin had immediate access to adenylate cyclase, and that the A_1 peptide alone was sufficient to activate the cyclase.

1.3.4 Models for the entry of the A_1 chain

As previously mentioned in order to activate adenylate cyclase all or part of subunit A must be transported to the cytoplasmic surface of the membrane. There have been several theories put forward as to how this might occur. The simplest is that suggested by van Heyningen and King (1975), who observed that subunit A is active alone with intact cells. They proposed that there is no specific mechanism of entry, but that a high local concentration of toxin bound to ganglioside would increase the chance of a few molecules entering by some random process. Once inside the cell the A_1-A_2 disulphide bond may be cleaved by the thiol:protein disulphide oxidoreductase enzyme, which is found associated with membranes (Moss *et al.*, 1980). The main argument against this is that proteins, especially hydrophilic ones, are not thought to cross cell membranes by themselves to enough of an extent to account for the observed activity of the toxin.

Another theory is that of Gill (1976a) who proposed that the five B subunits dissolved in the membrane forming a hydrophilic channel through which the A subunit could pass. Also experiments with lipid bilayers showed that the toxin could induce permeability changes, perhaps indicating the formation of a pore or channel (Moss *et al.*, 1977; Tosteson and Tosteson, 1978). However, this has been ruled out, in this form at least, since photoaffinity labelling experiments (Wisnieski and Bramhall, 1981) and x-ray diffraction analysis (Ribi *et al.*, 1988) shows no evidence

of membrane penetration by the B subunits. A modification of this theory is that the B subunits might combine with a preexisting carrier protein already present in the membrane thus forming the required channel (Gill, 1978).

Another possibility is that entry is by endocytosis, although the problem here is that the endocytosed protein is still separated from the cytoplasm by a membrane. However, there is little evidence for this even though some endocytosis has been observed (Manuelidis and Manuelidis, 1976; Hansson *et al.*, 1977). Toxin taken up in this way does not seem to be active with intact cells. Also, it would be expected that a protein binding tightly and in large amounts to the cell surface would be endocytosed at least to some extent. It is harder to show that the endocytosis is a prerequisite for activity.

It has also been suggested that the A_1 fragment traverses the membrane by direct interaction with the hydrophobic interior of the bilayer (Tomasi and Montecucco, 1981; Wisnieski and Bramhall, 1981). However, whether the whole of the A_1 chain has to penetrate the membrane is not very clear since small proteolytic fragments are claimed to show activity (Matuo *et al.*, 1976; van Heyningen and Tait, 1980). Van Heyningen (1977b) reported that cross-linked cholera toxin as well as native toxin could activate adenylate cyclase in intact cells, suggesting that the A_1 peptide does not necessarily dissociate from the rest of the toxin as it passes through the membrane. Most people assume the $5B\text{-}A_2$

component remains outside the cell (Wisnieski and Bramhall, 1981), although some cells, such as neuronal cells, take up the whole toxin (Joseph *et al.*, 1978).

1.4 Effects of cholera toxin

A diagrammatic representation of some of the reaction processes occuring in the intestinal cell is shown in Figure 1-2, and they are discussed in the following sections.

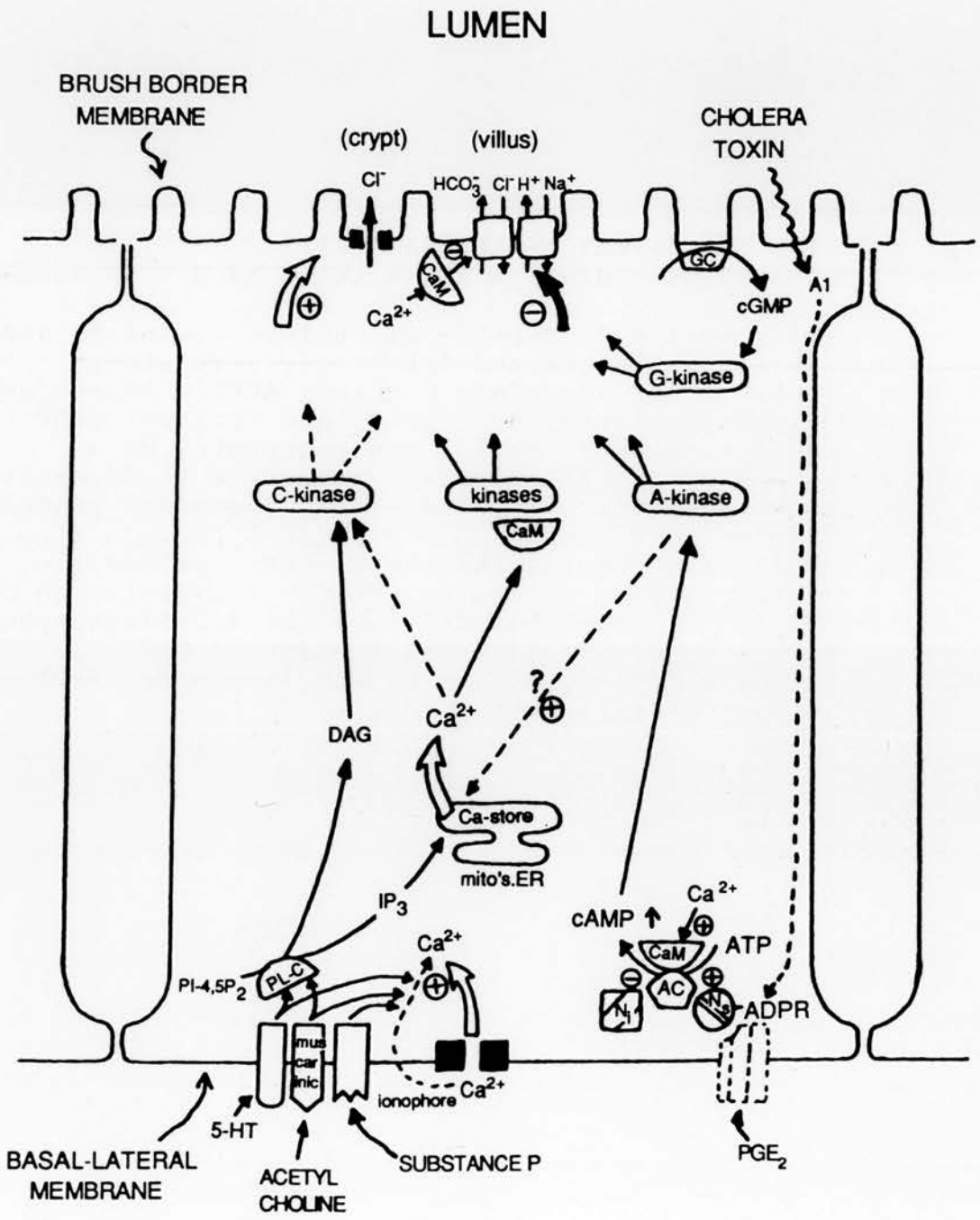
1.4.1 ADP-ribosylation and activation of adenylate cyclase

The enzyme adenylate cyclase is a complicated protein consisting of three components, namely the hormone receptor, the catalytic unit responsible for synthesizing cyclic AMP from ATP and the regulatory component that binds guanine nucleotides. This regulatory component has various names including GTP-binding protein, G protein (because it binds guanine nucleotides), N protein (because it binds nucleotides) and G/F protein (because it is responsive to both guanine nucleotides and fluoride). There are at least two types of regulatory proteins, one of which stimulates the catalytic subunit of adenylate cyclase, G_s, and another of which inhibits, G_i. Cholera toxin activates adenylate cyclase via G_s, while pertussis toxin activates via G_i.

The role of GTP in the regulation of adenylate cyclase was first recognized by Rodbell *et al.* (1971), who used it to restore hormone sensitivity to the cyclase in isolated membranes. Moss *et al.* (1976) discovered that cholera toxin

Fig. 1-2 The intestinal epithelial cell.
(Based on a diagram given to me by H R de Jonge)

A1 - active A_1 peptide of cholera toxin; N_s and N_i - stimulatory and inhibitory regulatory subunits of adenylate cyclase; ADPR - Adenosine diphosphoribose; AC - adenylate cyclase; cAMP - adenosine 3′,5′-cyclic monophosphate; GC - guanylate cyclase; cGMP - guanosine 3′,5′-cyclic monophosphate; A-kinase - cAMP-dependent protein kinase; G-kinase - cGMP-dependent protein kinase; C-kinase - protein kinase C; CaM - calmodulin; ER - endoplasmic reticulum; PL-C - phospholipase C; PI-4,5P_2 - phosphatidyl inositol 4,5-bisphosphate; IP_3 - inositol 1,4,5-trisphosphate; DAG - diacylglycerol; PGE_2 - prostaglandin E_2; 5-HT - 5-hydroxytryptamine.


LUMEN
BRUSH BORDER MEMBRANE
(crypt)
(villus)
CHOLERA TOXIN
Cl⁻
HCO3⁻ Cl⁻ H⁺ Na⁺
CaM
Ca2+
GC
cGMP
A1
G-kinase
C-kinase
kinases
CaM
A-kinase
DAG
Ca2+
?
Ca-store
mito's.ER
IP3
cAMP
Ca 2+
CaM
ATP
AC
ADPR
PI-4,5P2
PLC
Ca2+
muscarinic
ionophore
Ca2+
5-HT
BASAL-LATERAL MEMBRANE
ACETYL CHOLINE
SUBSTANCE P
PGE2

catalyzed the hydrolysis of NAD^+ to ADP-ribose and nicotinamide (NAD^+-glycohydrolase activity). Moss and Vaughan (1977) further demonstrated that the release of nicotinamide from NAD^+ was greatly stimulated by D- or L-arginine. ADP-ribosyl-L-arginine was formed as a product when L-arginine was present. In 1977 Levinson and Blume showed that cholera toxin activation of the cyclase was dependent on GTP as well as NAD^+.

Although much of the NAD^+-glycohydrolase activity of the crude toxin may be due to another enzyme (Tait and van Heyningen, 1978) it is clear that both NAD^+-glycohydrolase and ADP-ribosyl transferase activities are true properties of the A_1 peptide of the toxin. Reported K_M values for NAD^+ using artificial acceptors are 3.6 mM (Mekalanos *et al.*, 1979a) and 5 mM (Moss *et al.*, 1979).

Pfeuffer (1977) was the first to identify a GTP-binding protein of molecular mass 42 kDa using a photoreactive GTP analogue. This regulatory component could be separated from the catalytic unit by affinity chromatography on GTP-Sepharose. The cyclase depleted of this component did not respond to guanosine 5′-[β,γ-imido]triphosphate (Gpp(NH)p), a non-hydrolysable GTP analogue, or to fluoride ions, but response was restored when the isolated regulatory component was added. This showed that the ADP-ribosylation of this regulatory component by cholera toxin was the basis of activation of adenylate cyclase.

It was shown that when [adenylate-^{32}P]-NAD^+ was supplied, cholera toxin catalyzed the ADP-ribosylation of specific proteins (both soluble and membrane bound) in pigeon erythrocytes (Cassel and Pfeuffer, 1978; Gill and Meren, 1978; Gill, 1979). The principal ADP-ribosylated protein was the one of 42 kDa identified by Pfeuffer (1977).

As far as different tissues and cells are concerned, cholera toxin has been generally found to catalyze the ADP-ribosylation of proteins with a molecular mass of 42-56 kDa, some of which have been identified as the regulatory component of adenylate cyclase (for a general review see Enomoto and Gill, 1983).

The protein of 42 kDa now seems to be a subunit of G_s (Katada and Ui, 1982a). The two regulatory proteins, G_s and G_i, have been purified (Sternweis *et al.*, 1981; Bokoch *et al.*, 1983, 1984; Codina *et al.*, 1983, 1984) and appear to be similar both structurally and functionally. They both consist of $\alpha\beta\gamma$ heterotrimers (Hildebrandt *et al.*, 1984) of molecular mass 80-90 kDa (Bokoch *et al.*, 1984; Codina *et al.*, 1984). Their α (42-45 kDa for $G_{s\alpha}$ and 41 kDa for $G_{i\alpha}$) and $\beta\gamma$ (35 kDa for β and 5-10 kDa for γ) subunits are heterogeneous, but the heterogeneity of the $\beta\gamma$ subunits may be due to stable combinations of similar β subunits with different γ subunits (Neer and Clapham, 1988). Indeed, cells other than retinal cells have been found to contain two closely-related β subunits, a major 36 kDa form and a minor 35 kDa form (Roof *et al.*, 1985; Evans *et al.*, 1987),

whereas Evans *et al.* (1987) have defined two types of non retinal γ subunits. Therefore, the existence of at least two forms of β and γ subunits means that there are at least four possible stable β-γ combinations in non retinal cells. The α subunits of both proteins bind GTP (Northup *et al.*, 1982), whereas the β and/or γ subunits are probably responsible for anchoring the α subunit to the plasma membrane (Sternweis, 1986). Activation of either protein (G_s and G_i) is guanine nucleotide- and Mg^{2+}-dependent (Northup *et al.*, 1982; Hildebrandt and Birnbaumer, 1983), is also brought about by sodium fluoride in the presence of Mg^{2+} ions (Sternweis *et al.*, 1981) and appears to result in the dissociation of the proteins into separate α and $\beta+\gamma$ components (Hildebrandt *et al.*, 1983; Bokoch *et al.*, 1983; Northup *et al.*, 1983a; Kahn and Gilman, 1984a). G_s and G_i are substrates for bacterial toxin catalyzed NAD^+-dependent ADP-ribosylation (Fig. 1-3). The α subunit of G_s ($G_{s\alpha}$) is ADP-ribosylated by the A_1 peptide of cholera toxin. Specifically A_1 transfers the ADP-ribosyl group from NAD^+ to an arginine residue of $G_{s\alpha}$ (Gill and Meren, 1978; Cassel and Pfeuffer, 1978). Cassel and Selinger (1977) have shown that adenylate cyclase is active while GTP is bound to the GTP-binding component, but reverts back to an inactive state as GTP is hydrolysed to GDP by its inherent GTPase activity. The effect of cholera toxin is to inhibit this GTPase activity, thus prolonging the active state of the G protein (Cassel and Selinger, 1977). This stabilizes adenylate cyclase in an active conformation, producing increasing amounts of cyclic AMP.

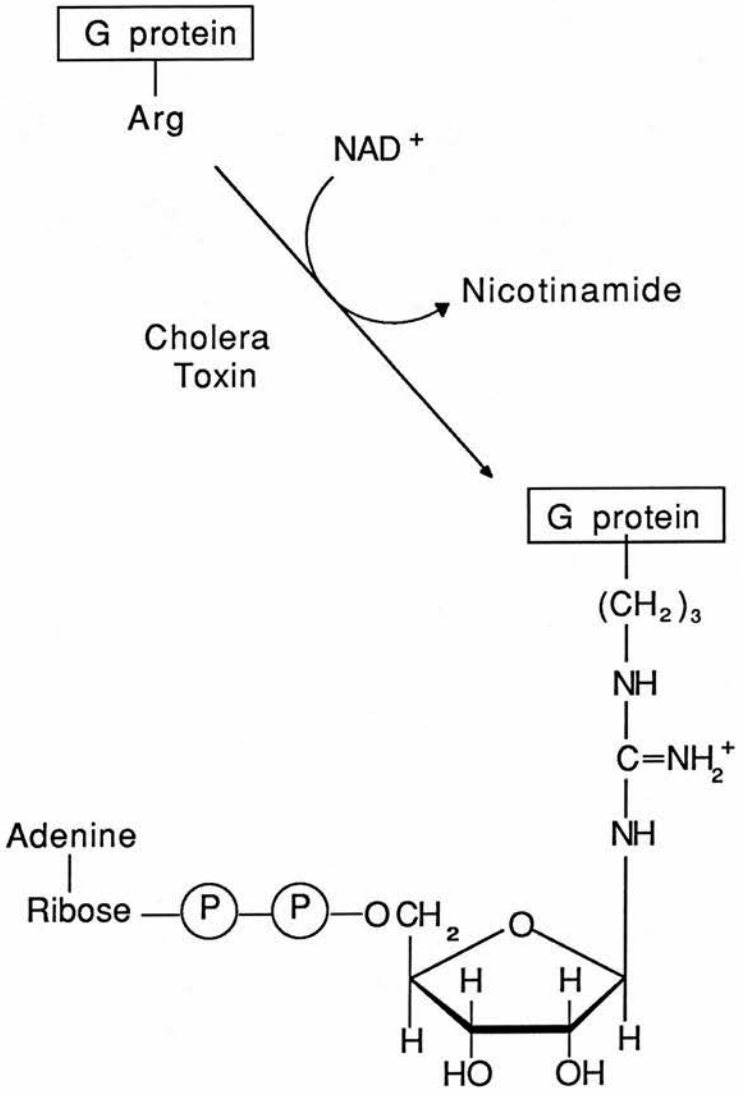


Fig. 1-3 **Mechanism for the ADP-ribosylation of the G protein, the regulatory component of adenylate cyclase, by cholera toxin.**

As well as ADP-ribosylation playing an important role in the activation of adenylate cyclase by certain bacterial toxins, it is becoming evident that it is also important in maintaining the level of cyclic AMP within the cell. In 1986, while looking at the ADP-ribosylation of adipocyte plasma membranes by cholera and pertussis toxins, Jacquemin *et al.* found endogenous ADP-ribosylation of G_s as a result of the sustained stimulation of G_i by adenosine during the incubation of control cells. Their results indicate that there may be a possible homeostatic mechanism in which the autonomous adjustment of the basal activity of G_s as a function of that of G_i , under the control of any feedback inhibitory ligands, ensures that there is a steady production of cyclic AMP in the cell.

Less progress has been made in the characterization of the catalytic component of adenylate cyclase. However, the purification of a protein of molecular mass 130-150 kDa has been reported (Coussen *et al.*, 1985; May *et al.*, 1985; Pfeuffer *et al.*, 1985; Yeager *et al.*, 1985; Smigel, 1986).

The theory of the sequence of events producing the hormonal stimulation of adenylate cyclase is shown in Figure 1-4 A,B and summarized as follows (Gilman, 1984a). The binding of the hormone, H, to its receptor, R_s , causes a conformational change in the receptor which is transmitted to G_s , making it susceptible to binding by GTP. When GTP binds, the G protein takes on a different conformation leading to the dissociation of the α subunit from the $\beta+\gamma$ subunits. The

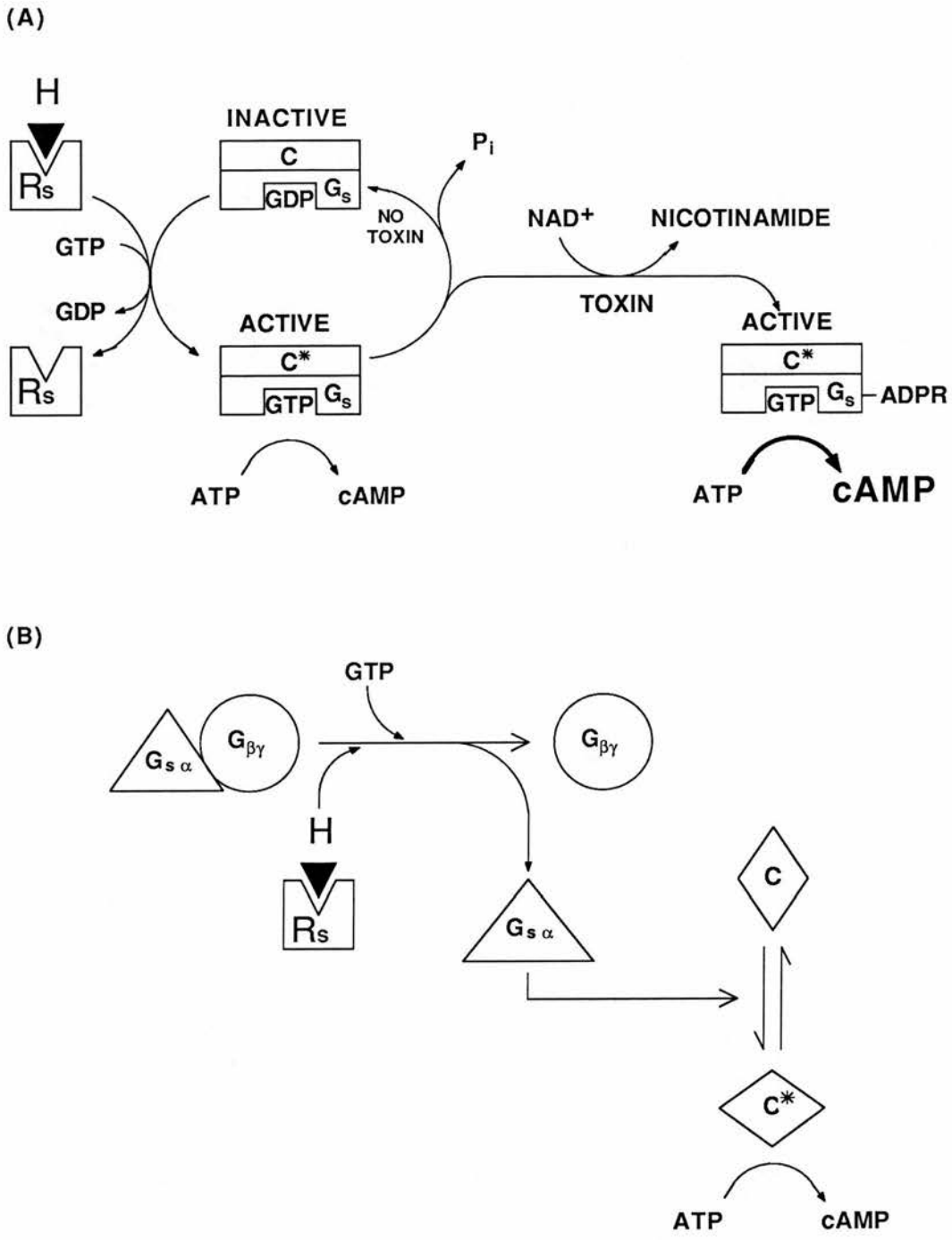


Fig. 1-4 Mechanism of activation of adenylate cyclase by cholera toxin.

(A) Scheme showing the activation of adenylate cyclase by a hormone, H, binding its receptor, R_s, and by cholera toxin (Adapted from van Heyningen, 1982).
(B) Scheme showing the interaction of the subunits of the G_s protein with the catalytic component, C, of adenylate cyclase to produce the active catalytic form, C* (Adapted from Gilman, 1984a).

GTP-occupied α subunit ($G_{s\alpha}$) can then bind and activate the catalytic subunit (Fig. 1-4 B), producing cyclic AMP from ATP. In fact it has been shown by Northup *et al.* (1983b) that the $\beta+\gamma$ subunits inhibit adenylate cyclase as a result of their interaction with the α subunit. Activation of the cyclase is terminated by the specific GTPase activity (Cassel and Selinger, 1976) which resides in the α subunit (Stadel and Lefkowitz, 1983; Gilman, 1984a,b; Schramm and Selinger, 1984), resulting in the re-association of the subunits and a return to the inactive state (Fig. 1-4 A). The effect of cholera toxin can then be simply explained by its ability to prolong the life of the activated $G_{s\alpha}$-GTP complex so that increasing amounts of cyclic AMP are produced, independently of stimulatory receptors and ligands (Cassel and Pfeuffer, 1978; Gill and Meren, 1978; Johnson *et al.*, 1980).

1.4.2 Cofactor requirements for ADP-ribosylation

As well as NAD^+ (Gill, 1975) and GTP (Gill, 1976b; Enomoto and Gill, 1979), a cytosolic factor with a molecular mass of about 20 kDa (Enomoto and Gill, 1979, 1980) was found to be required for the rapid ADP-ribosylation of the G_s protein. Once the G_s protein had been purified from various membranes (Northup *et al.*, 1980; Sternweis *et al.*, 1981; Hanski *et al.*, 1981) the requirements for cholera toxin-catalyzed ADP-ribosylation could be properly investigated. An intrinsic membrane protein that is required for the cholera toxin-dependent ADP-ribosylation of purified G_s (Schleifer *et al.*, 1982) has been purified about 2000-fold from cholate

extracts of rabbit liver membranes (Kahn and Gilman, 1984b). This protein, termed ARF (ADP-Ribosylation Factor), has a molecular mass of 21 kDa and was found to have a high-affinity binding site for guanine nucleotides (Kahn and Gilman, 1986). The binding of GTP or GTPγS (a non-hydrolyzable GTP analogue) to ARF is necessary for the activity of the cofactor (Kahn and Gilman, 1986). Kahn and Gilman (1984b) proposed that a $G_{s\alpha}$.ARF complex served as the cholera toxin substrate. However, it has recently been shown by Tsai *et al.* (1987) that bovine brain ARF enhances not only the ADP-ribosylation of $G_{s\alpha}$ but also $G_{s\alpha}$-independent cholera toxin-catalyzed reactions. These are the hydrolysis of NAD^+, the ADP-ribosylation of low molecular weight guanidino compounds such as agmatine, the ADP-ribosylation of several proteins unrelated to $G_{s\alpha}$ and the auto-ADP-ribosylation of the toxin A_1 peptide. These reactions as well as the ADP-ribosylation of ARF itself were stimulated by GTP or stable GTP analogues. These observations are consistent with the direct interaction of ARF with cholera toxin in a GTP-dependent manner enhancing the catalytic activity of the aforementioned reactions. Two soluble proteins (sARF1 and sARF2) from bovine brain that also enhance the $G_{s\alpha}$-dependent and -independent ADP-ribose transfer reactions catalyzed by the toxin have also been purified and characterized (Tsai *et al.*, 1988). As with the membrane ARF (mARF), both soluble factors are 19 kDa proteins dependent on GTP or GTP analogues for activity. It appears that these soluble factors, like mARF, directly activate cholera toxin in a GTP-dependent way.

A second guanine nucleotide binding site, termed S, was described in 1983 by Gill and Meren. They showed that the binding of nucleotide to S is promoted by a cytosolic protein factor, which they named CF. In 1987, Gill and Coburn showed that non-hydrolyzable GTP analogues have a stable effect on S allowing the CF-directed activation of S to be separated temporally from the assay of the effect of activated S on ADP-ribosylation rates. They believe ARF (Schleifer *et al.*, 1982; Kahn and Gilman, 1984b) and S are the same protein, but despite earlier suppositions (Gill and Meren, 1983; Kahn and Gilman, 1984b) that ARF/S interacts with G_s the data shows that the direct activation of cholera toxin by ARF/S is more likely, in agreement with Tsai *et al.* (1987, 1988).

1.4.3 Cyclic-nucleotide-dependent protein phosphorylation and intestinal secretion

The movement of electrolytes across the small intestinal epithelium (Kimberg, 1974; Field, 1976) which can be caused by the enterotoxins of *Vibrio cholerae* and *Escherichia coli* and hormones such as glucagon, vasoactive intestinal peptide (Schwartz *et al.*, 1974), vasopressin and several prostaglandins is mediated by at least three intracellular signals: (1) cyclic AMP, resulting from the increase in activity of adenylate cyclase by cholera toxin, which exerts its effect through the activation of intestinal cyclic-AMP-dependent protein kinase (Alhanaty and Shaltiel, 1979), (2) cyclic GMP, produced by guanylate cyclase in the brush border membrane and which activates cyclic-GMP-dependent

protein kinase (de Jonge, 1981), and (3) calcium, probably acting through calmodulin (Ilundain and Naftalin, 1979) or through a phospholipid-dependent (protein kinase C) protein kinase (de Jonge, 1983). Although there may be cyclic nucleotide- and Ca^{2+}-sensitive carriers operating in the basal-lateral membranes of intestinal cells (Brown *et al.*, 1983) and in the tight junctions (Bakker and Groot, 1983), the major secretory response is thought to occur in the brush border membrane (Frizzell *et al.*, 1979). The potential cyclic nucleotide- and Ca^{2+}-sensitive ion transport carriers in epithelial membranes include (1) an electroneutral Na^+-Cl^- cotransporter in intestinal brush border membranes (Frizzell *et al.*, 1979; Fan *et al.*, 1983). The nature of the coupling of the Na^+ to Cl^- transport is unclear. However, Fan *et al.* (1983) proposed, as did Liedtke and Hopfer (1982a,b), that rather than there being a direct coupling of Na^+ to Cl^- it might be Na^+/H^+ and Cl^-/OH^- (or HCO_3^-) antiporters which account for the comovements of Na^+ and Cl^- (an influx) across the villus cell brush border membrane. (2) A Cl^- channel in the brush border membrane, probably localized in crypt cells and maybe lower villus cells (Frizzell *et al.*, 1979; Welsh *et al.*, 1982). (3) transport systems occuring in the basal-lateral membranes which facilitate active Cl^- secretion, for example a coupled entry mechanism for Na^+-Cl^- (Frizzell *et al.*, 1979), the Na^+,K^+ pump (Hootman *et al.*, 1983), and Ca^{2+}-regulated K^+ channels (Brown *et al.*, 1983).

Since the biological effects of cyclic nucleotides may be mediated through the regulation of the activity of protein kinases (Kuo and Greengard, 1969; Shlatz *et al.*, 1978), and the cyclic-nucleotide-dependent protein kinases are integral components of plasma membranes in a wide variety of tissues (Rubin *et al.*, 1972; Ueda *et al.*, 1973; Forte *et al.*, 1975; Azhar and Menon, 1975), it seems likely that the phosphorylation and dephosphorylation of unique membrane proteins could provide the mechanism for the regulation of membrane function and structure. Indeed, there has been good evidence showing an effect of cyclic nucleotides on the phosphorylation of small intestinal brush border membranes (Lucid and Cox, 1972; de Jonge, 1976,1981; Shlatz *et al.*, 1978, 1979). Van Dommelen and de Jonge (1984) have shown evidence for a Cl^- channel in intestinal brush border membranes activated by the cyclic-AMP-dependent phosphorylation of one or more membrane proteins. In 1972 Lucid and Cox found that there was an enhanced incorporation of ^{32}P in brush border membranes after treatment with cholera toxin by an *in vivo* method. However, because they measured the total ^{32}P-incorporation by liquid scintillation counting it is impossible to say if any unique proteins are phosphorylated in the presence of cholera toxin. Later, de Jonge (1976) found that a protein of molecular mass 86 kDa was phosphorylated by both cyclic AMP- and cyclic GMP-dependent protein kinases in rat intestinal brush border membranes. However, he did not look at any effects on the basal-lateral membrane. Therefore, in 1978 Shlatz *et al.* looked to see if the purified plasma membranes of rat enterocytes (brush border versus basal-

lateral) had the necessary components for phosphorylation: an endogenous cyclic-nucleotide-dependent protein kinase, endogenous protein substrates, and a phosphoprotein phosphatase. They found that although both membranes were phosphorylated by an endogenous protein kinase and contain a phosphoprotein phosphatase, the brush border membranes were phosphorylated and dephosphorylated to a greater extent than the basal-lateral membranes. However, in contrast to these results Scalera *et al.* (1983) found no evidence for the cyclic-AMP-dependent phosphorylation of a brush border membrane protein, either by the perfusion of rat jejunum *in vivo* with [^{32}P]orthophosphate or by the *in vitro* phosphorylation of brush border membranes by [γ-^{32}P]ATP, both in the presence of cholera toxin. They, therefore, came to the conclusion that the alteration in NaCl absorption caused by cholera toxin was probably not mediated via cyclic AMP protein kinase activity, which would lead to an altered protein phosphorylation of brush border membranes.

Evidence has accumulated suggesting that calcium is a physiological regulator of intestinal electrolyte transport (Bolton and Field, 1977). Conditions which increase intracellular calcium, such as the use of the calcium ionophore A23187 (Frizzell, 1977), cause the stimulation of intestinal chloride secretion and inhibition of sodium chloride absorption. To the contrary, exposure to the calcium channel blocker verapamil, to decrease the intracellular calcium concentration, stimulates sodium chloride absorption (Donowitz and Asarkof, 1980).

Calmodulin is thought to be involved in these effects since the antipsychotic drug trifluoperazine (see section 1.4.4), which binds calmodulin tightly, inhibits the intestinal secretion caused by calcium ionophore A23187 (Smith and Field, 1980). On the basis of $^{22}Na^{+}$ and $^{36}Cl^{-}$ isotope flux experiments in rabbit brush border membrane vesicles, Fan and Powell (1983) have reported that Ca^{2+} and Ca^{2+}-calmodulin inhibit microvillar Na^{+}-Cl^{-} transport. Interestingly though, protein phosphorylation was not apparently involved in this regulatory mechanism. Having said this, the authors do speculate that the protein kinase-mediated phosphorylation of membrane proteins might be responsible for the inhibition of Na^{+}-Cl^{-} transport, since Taylor *et al.* (1981) have reported the Ca^{2+}/calmodulin-sensitive phosphorylation of rabbit ileal membrane proteins. Since calcium and calmodulin affect phosphorylation and the function of specific proteins in several other systems (Schulman and Greengard, 1980), it seems likely that Ca^{2+}/calmodulin-sensitive as well as cyclic-nucleotide-sensitive phosphorylation is a control mechanism for electrolyte transport.

1.4.4 Effect of antidiarrhoeal drugs

A certain class of drugs known as the phenothiazines, which are antipsychotic drugs, have been found to inhibit the diarrhoea caused by cholera toxin by mechanisms which are not yet fully understood. However, the main sites of action at which they are thought to affect membrane transport are adenylate cyclase, calmodulin and protein kinases.

As mentioned in the previous section the concentration of calcium has been implicated in the control of ion secretion and the acidic, heat stable, Ca^{2+}-binding protein calmodulin has been suggested to be important in mediating these events (Fan and Powell, 1983). Calmodulin appears to be able to bind four Ca^{2+} ions, and upon binding Ca^{2+} its conformation changes exposing hydrophobic regions (La Porte *et al.*, 1980) and increasing its helical content (Liu and Cheung, 1976). It is this conformational change which allows binding of the calmodulin-Ca^{2+} complex to specific sites on enzymes, thus increasing their activities by an unknown mechanism. The binding of Ca^{2+}-activated calmodulin activates a number of Ca^{2+}-dependent enzymes including a specific molecular form of phosphodiesterase (Uzunov and Weiss, 1972), adenylate cyclase (Amiranoff *et al.*, 1983), guanylate cyclase, (Ca^{2+}+Mg^{2+})-ATPase (Caroni and Carafoli, 1981), phospholipase A_2 (Wong and Cheung, 1979) and protein kinases (Srivastava *et al.*, 1979; Kennedy and Greengard, 1981). It has been shown that the phenothiazines bind to calmodulin in a Ca^{2+}-dependent manner with both a high affinity and specificity, and so this has been proposed as a mechanism of their action (Levin and Weiss, 1977, 1978; Weiss and Levin, 1978; La Porte *et al.*, 1980); there are one to three Ca^{2+}-dependent drug binding sites per calmodulin molecule. In fact a correlation between the high affinity for calmodulin and the antisecretory activity of many drugs has been shown.

There appear to be two kinds of interactions between the drugs and calmodulin: (i) a hydrophobic interaction between a lipophilic portion of the drug and a non-polar region of calmodulin and (ii) an electrostatic interaction between a positively charged amino group on the drug and a negatively charged acidic residue on calmodulin (Prozialeck and Weiss, 1982; Weiss *et al.*, 1982). The most potent inhibitors of calmodulin have a very large hydrophobic region, consisting of two aromatic rings, joined at one or two positions, a side-chain amino group that is at least four atoms removed from the aromatic ring structure and the addition of a moiety on the aromatic ring to increase its hydrophobicity (Prozialeck and Weiss, 1982; Weiss *et al.*, 1982).

The pharmacological actions of the phenothiazines on the central nervous system are quite varied; these include antipsychotic, sedative and extrapyramidal effects. These drugs also influence the neuroendocrine, gastrointestinal and cardiovascular systems, particularly when used in high concentrations (Weiss *et al.*, 1982). Since calmodulin influences a wide variety of cellular events, particularly nervous, muscular and secretory systems, its inhibition may provide a common mechanism for explaining some of the diverse biochemical actions of the drugs. Although the phenothiazine antipsychotics are among the most potent calmodulin inhibitors there are several other pharmacological classes of drugs that can also inhibit calmodulin (see Weiss *et al.*, 1982); these include α-adrenergic antagonists (Earl *et al.*, 1982), antimalarials

(Volpi *et al.*, 1981), cancer chemotherapeutic agents (Katoh *et al.*, 1981), antihistaminics (Weiss *et al.*, 1980), antidepressants (dibenzazepines) (Levin and Weiss, 1976), antianxiety agents (benzodiazepines) (Levin and Weiss, 1978; Weiss *et al.*, 1980) and antipsychotic drugs belonging to other chemical classes, particularly diphenylbutylpiperidines, the thioxanthenes and, to a lesser extent, the butyrophenones, dibenzazepines and dibenzodiazepines (Levin and Weiss, 1978; Weiss *et al.*, 1980).

However, since calmodulin has such a diverse regulatory action, how can a drug which inhibits calmodulin have a specific action? The answer could be that (i) the specificity with which calmodulin inhibitors act depends on drug disposition, localization, binding and transport across specialized membranes and (ii) maybe a different concentration of calmodulin is required to activate the different Ca^{2+}-dependent enzymes. If this is true then a calmodulin inhibitor might exert a greater effect on one calmodulin-dependent process than another.

Various drugs have been investigated both for their capacity as antidiarrhoeal agents and as inhibitors of calmodulin activity. Chlorpromazine, a phenothiazine, has been reported to inhibit the hormonal stimulation of adenylate cyclase in various tissues (Kakiuchi and Rall, 1968; Wolff and Jones, 1970; Osnes *et al.*, 1976). This prompted Lönnroth *et al.* (1977) to look at the effects of this drug on the cholera toxin activation of adenylate cyclase in

mouse thymocytes *in vitro* and cholera-toxin-induced intestinal secretion in mice *in vivo*. They found that chlorpromazine inhibited the formation of cyclic AMP, and prevented intestinal hypersecretion in response to cholera toxin. The decrease in cyclic AMP formation is not likely to be due to an increase in phosphodiesterase-induced cyclic AMP degradation, since the drug has been reported to inhibit rather than enhance the activity of phosphodiesterase in other systems (Wolff and Jones, 1970). Holmgren *et al.* (1978) examined the antisecretory activity of chlorpromazine more closely. They found that this drug inhibits the intestinal secretion caused by various diarrhoeagenic agents (*E. coli* heat-labile enterotoxin, prostaglandin E_1 and dibutyryl cyclic AMP) which are known to act through cyclic AMP (Pierce and Wallace, 1972; Kimberg, 1974; Field, 1976), and also turns off already established secretion without appearing to have any effect on absorption. They also found that these effects were associated with the marked inhibition of adenylate cyclase as well as protein kinase activities of mouse small intestinal epithelial membranes. Fluoride-stimulated adenylate cyclase activity was also inhibited, whereas basal activity was relatively unaffected. Clinical trials came in 1979 when Rabbani *et al.* found that chlorpromazine effectively reduced the fluid-loss in eleven cholera patients with severe purging. However, they do say that this drug does have some undesirable side-effects, particularly lowered blood-pressure, and so must be investigated further. After all the drug could be potentially dangerous when given to dehydrated patients and

patients undergoing rehydration by oral replacement in a somewhat less supervised setting. After finding that trifluoperazine, a phenothiazine, and several other related drugs which bind to calmodulin inhibit the Cl^- ion secretion associated with the diarrhoea induced by agents such as cholera toxin, prostaglandins and vasoactive intestinal peptide (Ilundain and Naftalin, 1979; Sandhu *et al.*, 1979; Smith and Field, 1980), more intensive work was carried out investigating the relationship between the effect of drugs on calmodulin binding and antisecretory activity. Prozialeck and Weiss (1982) looked at the abilities of various phenothiazine derivatives to inhibit the calmodulin-induced activation of phosphodiesterase *in vitro*, whereas Lönnroth *et al.* (1980) and Zavecz *et al.* (1982) investigated the effect of various phenothiazines and related drugs, having different binding capacities to calmodulin, for intestinal antisecretory activity. The results showed a positive correlation between the affinity of the drugs tested for binding to calmodulin and their ability to inhibit intestinal fluid secretion.

Thus, the modification of calmodulin activity might explain the pharmacology of drugs which are currently in use or suggest novel approaches for pharmacologically regulating various physiological processes (Weiss *et al.*, 1982).

1.5 Other toxins

There are three main ways in which bacterial toxins show similarities: in their two-component structure, in their binding to the cell and in their mechanism of action. Examples of toxins affecting eukaryotic cells by ADP-ribosylation, like cholera toxin, are diphtheria toxin, pertussis toxin, the heat labile toxin of *E. coli* and exotoxin A of *Ps. aeruginosa*. These and the two neurotoxins, tetanus and botulinum, are discussed in the following sections. Similarities between the bacterial toxins are shown in Table 1-1.

1.5.1 *E. coli* heat-labile toxin

Certain entero-pathogenic strains of *Escherichia coli* secrete a heat-labile protein toxin (LT) which activates adenylate cyclase and induces diarrhoea in exactly the same way as cholera toxin. *E. coli* toxin has the same subunit structure as cholera toxin (Clements *et al.*, 1980), produces a peptide similar to A_1 and catalyzes the ADP-ribosylation of a susceptible arginine on G_s (Evans *et al.*, 1972; Gill *et al.*, 1981; Moss and Vaughan, 1981). Subunits of both toxins cross-react immunologically and are highly homologous (Moseley and Falkow, 1980; Spicer *et al.*, 1981; Spicer and Noble, 1982; Dykes *et al.*, 1985). There seems to be little doubt that the two toxins are evolutionarily related.

1.5.2 Diphtheria toxin

Diphtheria toxin is synthesized and secreted by *Corynebacterium diphtheriae* as a single polypeptide chain

Table 1-1 **The component structure of various toxins** (adapted from van Heyningen, 1984)

Toxin	Mol.wt. of toxin	Active component			Binding component		
		Structure	Mol.wt.	Target	Structure	Mol.wt.	Target
Affecting adenylate cyclase							
Cholera	82000	Subunit A =A1 peptide +A2 peptide	27000 22000 5000	G_s protein of adenylate cyclase	Five B subunits	11600 each	Ganglioside G_{M1}
E.coli heat labile	91000	Subunit A =A1 peptide +A2 peptide	30000 25000 5000	G_s protein of adenylate cyclase	Five B subunits	11800 each	Ganglioside G_{M1} and glycoproteins
Pertussis	117000	S-1	28000	G_i protein of adenylate cyclase	S-2 S-3 Two S-4 S-5	23000 22000 11700 9300 each	Not known, may be a glycoprotein
Affecting protein synthesis							
Diphtheria	62000	A chain	24000	EF2 (diphthamide)	B chain	38000	Glycoprotein
Ps.aeruginosa exotoxin A	71000	A fragment	27000	EF2 (diphthamide)	B fragment	45000	Has no clear function
Neurotoxins							
Tetanus	150000	L chain	50000	No clear function	H chain	100000	Ganglioside G_{T1b}, G_{D1b}
Botulinum	150000	L chain	50000	No clear function	H chain	100000	Not known, may be a glycolipid

that can be cleaved proteolytically into two chains (fragments A and B). Diphtheria is an upper respiratory infection caused by bacteria lodged in the throat or nasopharynx where they cause the formation of a pseudomembrane over the pharynx, nasopharynx and upper trachea. This may lead to death by suffocation. In addition to the infection, muscle weakness and lethargy are observed, and death is often ascribed to heart failure.

Fragments A and B are held together by a single disulphide bond and both the intact and cleaved toxin are toxic. Fragment A catalyzes the ADP-ribosylation of elongation factor 2 (EF-2), thus inhibiting ribosomal protein synthesis (Honjo *et al.*, 1968; Collier and Cole, 1969; van Ness *et al.*, 1980a). As with G_s, EF-2 has GTPase activity which is destroyed by ADP-ribosylation, preventing protein synthesis and leading to cell death (Honjo *et al.*,1968). Unlike cholera toxin, diphtheria toxin catalyzes the ADP-ribosylation of diphthamide, a modified histidine (2-[3-carboxyamido-3-(trimethylammonio)-propyl]histidine) which has so far been only found in EF-2 (van Ness *et al.*, 1980a,b).

1.5.3 *Ps. aeruginosa* exotoxin A

Pseudomonas aeruginosa infections often follow wounding or burning. Exotoxin A of the bacterium shares strong analogies with diphtheria toxin (Vasil *et al.*,1977). The structure of the protein has some similarities to that of diphtheria toxin although they do not cross-react

immunologically and the toxin also catalyzes the ADP-ribosylation of EF-2 (Iglewski and Kabat, 1975; Iglewski *et al.*, 1977).

1.5.4 Pertussis toxin

Lymphocytosis promoting factor (LPF) is the major exotoxin secreted by *Bordetella pertussis*, the pathogenic bacterium causing whooping cough (Robinson *et al.*, 1985). Although the bacterium secretes many other toxins, LPF is the most important virulence factor and as such is more commonly referred to as pertussis toxin. It is also known as islet-activating protein (IAP) because of its unique action in enhancing the insulin secretory responses of pancreatic islet cells (Robinson *et al.*, 1985). The characteristic cough of this disease is caused by the bacteria colonizing the ciliated mucosa of the upper respiratory tract. Pertussis toxin is a hexamer (117 kDa) of five dissimilar peptides (Tamura *et al.*, 1982), having the same A(active)-B(binding) structure as the other toxins. It catalyzes the ADP-ribosylation of a 41 kDa protein (Katada and Ui, 1982a), which correlates with the pertussis-toxin-induced activation of adenylate cyclase resulting in an increase in cyclic AMP (Katada and Ui, 1982b; Murayama and Ui, 1983; Kurose *et al.*, 1983). It is now known that the toxin acts by ADP-ribosylating the inhibitory guanine-nucleotide-binding protein of adenylate cyclase, G_i (Ui, 1984). ADP-ribosylation inactivates G_i thereby blocking the inhibition of adenylate cyclase activity (Katada and Ui, 1982b; Bokoch *et al.*, 1983; Codina *et al.*, 1983). The amino acid that is modified has

been reported to be cysteine (West *et al.*, 1985; Lobban and van Heyningen, 1988).

1.5.5 Tetanus toxin

Tetanus toxin is the neurotoxin secreted by *Clostridium tetani* that is responsible for the dramatic muscular spasms of the disease tetanus. Although this disease has been known since the earliest days of medicine, relatively very little is known about it in comparison to other bacterial toxins. This is probably due to the fact that the target of the toxin, the nervous system, is very complicated and is itself not fully understood at the molecular level.

The first stage of the intoxication process is thought to be the diffusion of the toxin through the extracellular fluid from its point of entry (for example a cut) to the neuromuscular junction, where it is taken up. Following this internalization, the toxin is transported by retrograde axonal transport (Price *et al.*, 1975) to the cell body, from where it migrates trans-synaptically until it reaches the inhibitory synapses of the spinal cord or brain stem. The toxin is thought to act by the presynaptic blocking of the release of inhibitory neurotransmitters, especially γ-aminobutyric acid (Curtis *et al.*, 1973; Davies and Tongroach, 1979) and glycine (Curtis and de Groat, 1968), from these synapses. This results in the uninhibited firing of motor neurones, which leads to the characteristic spastic paralysis of the disease.

1.5.6 Botulinum toxin

The various strains of *Clostridium botulinum* produce at least eight different toxins. Seven of the eight toxins (A, B, C1, D, E, F and G) are neurotoxic and block the release of neurotransmitters at cholinergic but also at various other synapses by a still unknown mechanism (Habermann and Dreyer, 1986), resulting in the characteristic flaccid paralysis of this disease. Botulinum C2 toxin is not neurotoxic but cytotoxic and acts by ADP-ribosylating isolated actin (Aktories *et al.*, 1986a,b) and actin in intact cells (Reuner *et al.*, 1987), reducing the ability of the microfilament protein to polymerize (Aktories *et al.*, 1986a). It has recently been shown that certain strains of *Cl. botulinum* type C produce a second ADP-ribosyltransferase, C3, which is clearly distinct from the C2 toxin (Aktories *et al.*, 1987). The substrate of this C3 toxin appears to be 21-24 kDa guanine-nucleotide-binding protein(s) (Aktories and Frevert, 1987; Rösener *et al.*, 1987) found in various cell types (Aktories *et al.*, 1987). Botulinum neurotoxin D (Ohashi and Narumiya, 1987) and C1 (Ohashi *et al.*, 1987) have also been reported to be able to ADP-ribosylate an eukaryotic 21 kDa GTP-binding protein, which is supposedly involved in the toxin inhibition of exocytosis processes. However, Rösener *et al.* (1987) suggest that the ADP-ribosylating activities of the C1 and D neurotoxins are merely due to contamination by C3.

1.6 Aims of project

Over the past two decades, there has been a great increase in our knowledge of the molecular mechanisms of the ADP-ribosylating enterotoxins, such as cholera toxin (Gill, 1975; Moss *et al.*, 1976) and the heat-labile toxin of *Escherichia coli* (Moss and Richardson, 1978; Gill and Richardson, 1980), as activators of adenylate cyclase (Sharp and Hynie, 1971; de Jonge, 1975; Enomoto and Gill; 1983). However, the toxins are active in essentially all eukaryotic cells, and just about all of this work has been carried out on cells other than those of the intestine (Gill and Meren, 1978; Cassel and Pfeuffer, 1978; Northup *et al.*, 1980; Beckner and Blecher, 1981; Raufman and Cosowsky, 1987), where the toxin exerts its effect during the disease.

Interestingly, there is a problem in the activation of adenylate cyclase by cholera toxin, which is that the toxin binds to the brush border membrane of the intestinal epithelial cell (Walker *et al.*, 1974; Critchley *et al.*, 1981), while the catalytic component of adenylate cyclase is reported to be located at the basal-lateral membrane (Murer *et al.*, 1976; Walling *et al.*, 1978), i.e. on the other side of the cell. Using [adenylate-^{32}P]NAD^{+}, Domínguez *et al.* (1985) have shown that cholera toxin catalyzes the ADP-ribosylation of three proteins of molecular mass 40 kDa, 45 kDa and 47 kDa located in the brush border membrane of rabbit small intestinal epithelial cells. However, they could not demonstrate any similar effect with basal-lateral membrane proteins. They also showed that the activation of

adenylate cyclase by cholera toxin occured only with a crude plasma membrane preparation, while neither brush border nor basal-lateral membranes alone were responsive to the toxin. These results suggest that the modification of proteins at the brush border membrane (i.e. ADP-ribosylation) is a requirement for the activation of adenylate cyclase at the basal-lateral membrane. In contrast though, when fluoride ions were used as the activator, adenylate cyclase was activated in basal-lateral membranes, and to a greater extent than in crude plasma membranes (Domínguez *et al.*, 1985). Since sodium fluoride and cholera toxin both activate via the regulatory component of adenylate cyclase (Ross and Gilman, 1980), it would seem impossible for the activation by fluoride to rise at the same time as the activation by toxin falls during the preparation of basal-lateral membranes from crude plasma membranes.

Research has also been carried out on the phosphorylation of certain proteins which are found in the brush border and basal-lateral membranes. In 1976 de Jonge described the cyclic AMP- and cyclic GMP-dependent phosphorylation of a brush border protein, from rat intestine, with an apparent molecular mass of 86 kDa. However, he did not undertake any studies with purified basal-lateral membranes.

The need for a good, effective pharmacological agent to safely inhibit the intestinal secretion caused by cholera toxin is obviously of extreme importance. Even a partially effective antisecretory compound would be of considerable

value. Although oral replacement therapy with sugar-electrolyte solutions is almost 100% effective in cases where patients are suffering from mild or moderate dehydration, it often fails when the secretion is very intense (Nalin *et al.*, 1968; Nalin and Cash, 1971). An agent that could reduce the volume of fluid secreted by as much as 30%, for example from 1000 ml to 700 ml per hour, might make the difference between success and failure in the oral treatment of cholera. The phenothiazine antipsychotic drugs, for example chlorpromazine, have been found to inhibit the diarrhoea caused by cholera toxin by mechanisms which are not yet fully known (Lönnroth *et al.*, 1977; Holmgren *et al.*, 1978; Rabbani *et al.*, 1979), but are thought to affect membrane transport via calmodulin, adenylate cyclase and protein kinases. Since the phenothiazines and other related drugs have been shown to be effective in reducing the fluid loss, characteristic of cholera and other diarrhoeal diseases (Holmgren *et al.*, 1978; Lönnroth *et al.*, 1980; Zavecz *et al.*, 1982), it is necessary to find out more about their mechanisms of action with the long term view to the rational design of better antidiarrhoeal drugs, which lack the sedative action of drugs like chlorpromazine.

The first stage of the project involved obtaining intestinal cells from rabbits, preparing a purified plasma membrane fraction, separating brush border membranes from basal-lateral membranes and then performing biochemical experiments on them. Initially, the ADP-ribosylation of

proteins in each membrane fraction was investigated. This was to determine which proteins are labelled, whether the labelling is unique to a particular membrane and whether the major protein labelled has the same molecular mass as the regulatory component of adenylate cyclase. The effect of cholera toxin and sodium fluoride on the activity of adenylate cyclase, in each membrane fraction, was then investigated. This was to see if activation is greater in one membrane fraction compared to the other, and if the pattern of activation in the fractions is the same with both cholera toxin and sodium fluoride (sodium fluoride, a known potent activator of adenylate cyclase, was used as a comparison to the cholera-toxin-induced activation). The next stage was to identify which proteins are phosphorylated and in which membrane fraction this occurs, and to see if this phosphorylation correlates with the increase in cyclic AMP brought about by the activation of adenylate cyclase by cholera toxin.

The effect of drugs, with known antidiarrhoeal and anti-calmodulin activities, on the activation of adenylate cyclase and ADP-ribosylation was investigated. This was to determine the correlation between the affinity of the drugs for calmodulin, their antisecretory activity and their effects on adenylate cyclase activity and the ADP-ribosylation of specific membrane proteins.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials

Cyclic [8-^{3}H]AMP and [α-^{32}P]ATP were purchased from Amersham International.

[γ-^{32}P]ATP and [adenylate-^{32}P]NAD^{+} were from New England Nuclear.

Adenosine deaminase (type II), cyclic AMP (sodium salt), ADP (di-monocyclohexylammonium salt, grade V), ATP (disodium salt, grade II), BSA (98-99% albumin), cholera toxin, chromatographic alumina (neutral, type WN-3, activity grade 1), creatine phosphate (disodium salt hydrate, sigma grade 98-100%), creatine phosphokinase (type I), cytochrome c (type VI), dithiothreitol, GTP (type II-S), INT, D-mannitol, Mops, NADPH (tetrasodium salt, type I), ouabain (Strophanthin G octahydrate), saponin, sigma 104 phosphatase substrate, sodium succinate, sucrose and thymidine were from Sigma Chemical Co.

Glucose GOD-perid reagent and NAD^{+} (Grade 1, 100%) were from Boehringer Mannheim.

Hepes and isoniazid were from BDH chemicals.

3-Isobutyl-1-methylxanthine (Gold label) was from Aldrich Chemical Co.

The following materials, used for preparing the membrane samples for examination by electron microscopy, were kindly supplied by Dr D J S Hulmes of the Biochemistry Department, University of Edinburgh: sodium cacodylate, propylene oxide, paraformaldehyde, glutaraldehyde, osmium tetroxide, araldite CY212, dodecenyl succinic anhydride and benzyldimethylamine.

All other reagents were of analytical grade.

2.2 Methods

2.2.1 Preparation of membrane fractions

2.2.1.1 General method

Brush border membranes and basal-lateral membranes were prepared from intestinal epithelial cells as described below and as summarized in Figure 2-1.

One female white New Zealand rabbit was sacrificed by cervical dislocation and the jejunum and ileum removed. After cutting the intestine into smaller sections, each piece was thoroughly rinsed with isotonic saline (0.9% sodium chloride) and cut open along the mesenteric border. Each piece was gently scraped with a glass slide to remove the epithelial cells. After weighing the cells they were suspended in approximately 20 vol. 50 mM-D-mannitol, 5 mM-Hepes, pH 7.5, 0.25 mM-$MgCl_2$, (Buffer I), and homogenized with 20 strokes of a motor driven Potter-Elvehjem homogenizer. All manipulations were carried out on ice. Brush border and basal-lateral membranes were prepared by a modification of the method of Murer *et al.* (1976). After the removal of an aliquot for marker enzyme assays, the homogenate was centrifuged at 300g (Beckman JA-14 rotor, $r_{av.}$ 9.5 cm) for 10 min to remove whole cells and cell debris. This step was repeated. The resulting supernatant was centrifuged at 2,000g (JA-14 rotor) for 10 min to remove any nuclei and cell debris. The supernatant obtained from this stage was centrifuged at 9,750g (JA-14 rotor) for 10 min to remove the mitochondria. A brown pellet was produced with a fluffy white top layer. The supernatant was swirled

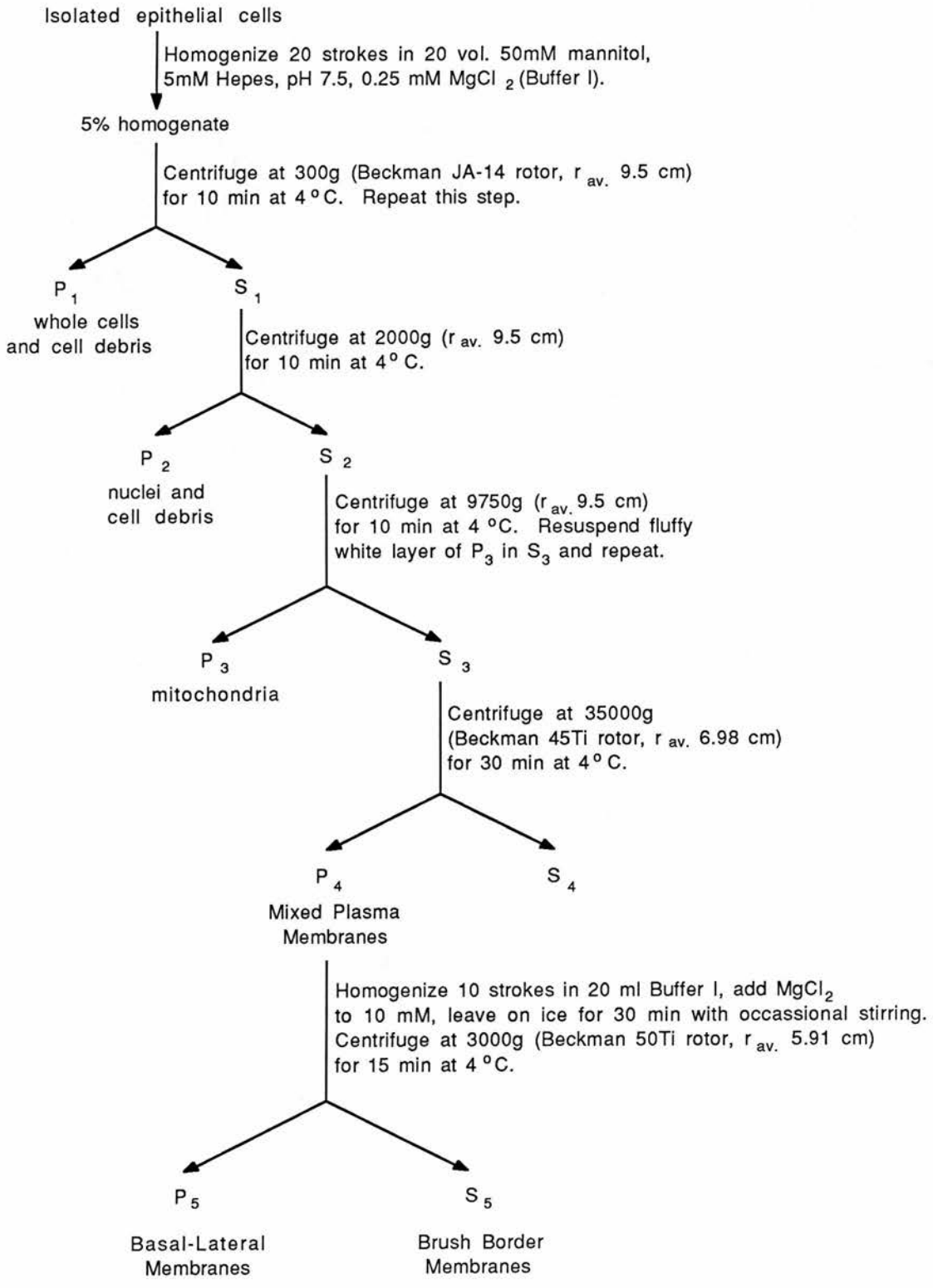


Fig. 2-1 Scheme for preparation of membrane fractions

The pellets and supernatants produced at each stage, n, of centrifugation are represented by P_n and S_n.

gently to remove this layer from the pellet and homogenized with 5 strokes. This step was then repeated. The pellet produced by centrifuging the resulting supernatant at 35,000g (Beckman 45Ti rotor, $r_{av.}$ 6.98 cm) for 30 min consisted of a mixture of brush border and basal-lateral membranes (the mixed plasma membrane fraction). The supernatant produced at this stage, S_4 (see Fig. 2-1), was also retained for resuspending the final membrane pellets, in order to provide any necessary cytosolic factors. The mixed plasma membrane fraction was used for preparing the two types of membrane by a Mg^{2+}-precipitation technique based on the method of Schmitz *et al.* (1973). The pellet was homogenized with 10 strokes in 20 ml of Buffer I, an aliquot for marker enzyme and experimental assays was removed, and this was followed by the addition of 1 M-$MgCl_2$ to a final concentration of 10 mM, and left on ice for 30 min with occassional stirring. An increase in turbidity was observed since the addition of $MgCl_2$ causes the aggregation of basal-lateral membranes, but not brush border membranes, into large particles which sediment at lower gravitational forces, probably because of electrostatic interactions between the cation and membrane surface charges. Basal-lateral membranes were then obtained by centrifugation at 3,000g (Beckman 50Ti rotor, $r_{av.}$ 5.91 cm) for 15 min, whilst the brush border membranes were obtained by centrifuging the resulting supernatant at 35,000g (50Ti rotor) for 30 min. Both pellets were then washed with 25 ml 1 mM-EDTA to remove excess Mg^{2+} ions, pelleted by centrifugation at 35,000g (Beckman 50.2Ti rotor, $r_{av.}$ 8.12 cm) for 30 min and finally

resuspended in 80% 1 mM-Hepes, pH 7.5, 0.1 M-D-mannitol, (Buffer II) / 20% S_4. Both of the aliquots removed from the homogenate and mixed plasma membrane fractions for marker enzyme assays were also pelleted at 35,000g (50Ti rotor) for 30 min and resuspended in 80% Buffer II / 20% S_4. Membranes were suspended at a concentration of 3-5 mg/ml, quick-frozen in liquid nitrogen, and stored at -70°C.

2.2.1.2 Determination of protein

Protein was determined by a modification of the method of Bradford (1976). A stock solution was prepared by dissolving 100 mg of coomassie brilliant blue G in 50 ml 95% ethanol and adding 100 ml 85% orthophosphoric acid. 18 ml of this stock solution was diluted to 100 ml with double-distilled water, and filtered through Whatman No.1 filter paper.

For protein estimation 0.5 ml of the membrane sample (containing 10-50 μg of protein) was added to 2.5 ml of the diluted Bradford protein reagent and the absorbance at 595 nm measured after 30 min. A standard curve was constructed using bovine serum albumin as the standard.

2.2.1.3 Marker enzyme assays

In order to determine the purity of the membrane fractions produced in section 2.2.1.1, marker enzyme assays, detailed below, were carried out. Table 2-1 summarizes the location of these enzymes in the cell. No golgi marker enzyme assay was determined. The linearity of all the assays was determined using purified commercial enzyme preparations or freshly prepared mitochondria.

Table 2-1 Cellular location of marker enzymes

EC no. - Enzyme Commission number; BBM - Brush Border Membrane; BLM - Basal-Lateral Membrane; LYS - Lysozyme; MITO - Mitochondria; ER - Endoplasmic Reticulum

Enzyme	EC no.	Location	Reference
Sucrase (Sucrose α-glucosidase)	3.2.1.48	BBM	Mircheff and Wright (1976)
(Na^+-K^+)ATPase (activity measured by its K^+-dependent phosphatase activity)	3.6.1.37	BLM	Mircheff and Wright (1976) and Avruch and Wallach (1971)
Alkaline phosphatase	3.1.3.1	BBM (and BLM)	Mircheff and Wright (1976)
Acid phosphatase	3.1.3.2	LYS	Gianetto and de Duve (1955)
Succinate dehydrogenase	1.3.99.1	MITO	Green *et al.* (1955)
NADPH-cytochrome c reductase	1.6.2.4	ER	Sottocasa *et al.* (1967)

2.2.1.3.1 Sucrase

Sucrase was assayed according to the method of Dahlqvist (1968). The assay mixture consisted of 28 mM-sucrose, 50 mM-NaH_2PO_4/Na_2HPO_4, pH 6.4, and 0.001-0.020 mg membrane protein: the diluted enzyme solution should contain less than 0.01 units/ml of sucrase activity (Dahlqvist, 1968), where 1 unit is equivalent to 1 μmol product produced per min. The reaction was started by the addition of the substrate to give a reaction volume of 0.2 ml, and the mixture was incubated for exactly 60 min at 37°C. The assay was also performed in the presence of 0.1% (w/v) saponin to disrupt the membrane vesicles, which might be present both in the right-side-out and right-side-in orientation, thus preventing the enzyme from being masked from its substrate. However, it is generally thought that membranes produced by divalent cation precipitation techniques are obtained as right-side-out orientated vesicles (Haase *et al.*, 1978). All solutions were preincubated for 5 min prior to the addition of the substrate. The reaction was stopped by the addition of 50 μl 0.5 M-Tris/HCl, pH 7: tris is only one of a large number of polyalcohols that inhibit disaccharidase activity (Dahlqvist, 1968). The glucose produced was quantified with glucose GOD-Perid reagent (Werner *et al.*, 1970). After centrifuging the samples at 9,000g ($r_{av.}$ 4.62 cm) for 5 min in a MSE Micro Centaur, 20 μl of the reaction mixture was added to 1 ml of the Perid reagent, and the solution incubated for 30 min at 25°C and the absorbance at 610 nm measured within a further 30 min. A glucose standard curve was constructed in order to determine the

quantity of glucose produced.

2.2.1.3.2 (Na$^+$-K$^+$)ATPase

(Na$^+$-K$^+$)ATPase was assayed by its ouabain-sensitive, K$^+$-dependent phosphatase activity, according to the method used by Murer *et al.* (1976) and adapted by Colas and Maroux (1980). The assay mixture contained 25 mM-Tris/HCl, pH 7.6, 5 mM-$MgSO_4$, 2.5 mM-EDTA, 45 mM-KCl, 0.05-0.15 mg membrane protein and 5 mM-p-nitrophenylphosphate (Sigma 104 phosphatase substrate), and was incubated for 30 min at 37°C in the presence and absence of 1 mM-ouabain. Ouabain was solubilized prior to adding to the assay mixture by warming at 37°C in 50 mM-Tris buffer, pH7.6, for 3 hr. The assay was performed in the presence and absence of 0.1% (w/v) saponin. The reaction was initiated by the addition of the p-nitrophenylphosphate to give a reaction volume of 1.2 ml, and stopped by adding 0.2 ml 3 M-NaOH. All solutions were preincubated for 5 min prior to the addition of the substrate, except for the membranes which were preincubated with the ouabain for 30 min. After centrifuging the samples at 9,000g (MSE Micro Centaur) for 5 min, the amount of p-nitrophenol released was determined by measuring the absorbance at 410 nm (the molar extinction coefficient for p-nitrophenol is 18300 M^{-1} at 410 nm). Ouabain-sensitive K$^+$-phosphatase activity was then determined by subtracting the value obtained in the presence from that in the absence of ouabain.

2.2.1.3.3 Alkaline phosphatase

Alkaline phosphatase activity was determined by the production of p-nitrophenol from p-nitrophenolphosphate (Mircheff and Wright, 1976). The assay mixture consisted of 40 mM-Tris/HCl, pH 9.0, 5 mM-$MgCl_2$, 0.25 mM-$CaCl_2$, 0.2 mM-$ZnCl_2$, 0.001-0.040 mg membrane protein and 5 mM-p-nitrophenylphosphate, and was incubated for 30 min at 37°C in both the presence and absence of 0.1% (w/v) saponin. The reaction was initiated by the addition of the substrate to give a reaction volume of 1 ml, and stopped by adding 3 ml 1M-NaOH. All solutions were preincubated for 5 min prior to the addition of the substrate. Samples were centrifuged at 9,000g (MSE Micro Centaur) for 5 min and the amount of p-nitrophenol produced determined by measuring the absorbance at 410 nm.

2.2.1.3.4 Acid phosphatase

Acid phosphatase activity was determined by the method of Michell *et al.* (1970), which involves the production of p-nitrophenol from p-nitrophenolphosphate. The assay mixture contained 50 mM-CH_3COONa buffer, pH 5.0, 4 mM-NaEDTA, 0.001-0.020 mg membrane protein and 10 mM-p-nitrophenylphosphate, and was incubated for 30 min at 37°C in both the presence and absence of 0.1% (w/v) saponin. NaEDTA was added to inhibit alkaline phosphatase, as suggested by Hübscher and West (1965). The reaction was initiated by the addition of the substrate to give a reaction volume of 1 ml, and stopped by adding 2 ml 0.1 M-NaOH. All solutions were preincubated for 5 min prior to

the addition of the substrate. Samples were centrifuged at 9,000g (MSE Micro Centaur) for 5 min and the amount of p-nitrophenol produced determined by measuring the absorbance at 410 nm.

2.2.1.3.5 Succinate dehydrogenase

Succinate dehydrogenase activity was measured by the reduction of 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl-tetrazolium hydrochloride (INT) as described by Pennington (1961), except for two modifications recommended by Porteous and Clark (1965): the assay was performed in the presence of 2 mM-EDTA and the complete system was kept at 0°C for 30 min prior to the assay. Porteous and Clark (1965) found that in the absence of EDTA reaction velocities were proportional to the amount of enzyme only above a certain minimum amount of enzyme and that below this quantity no enzymic activity was detected. However, with EDTA a linear plot of activity against protein content of the reaction mixture was obtained and this plot extrapolated to zero activity at zero enzyme concentration. The assay mixture contained 50 mM-K_2HPO_4/KH_2PO_4, pH 7.4, 50 mM-sodium succinate, 25 mM-sucrose, 2 mM-EDTA, 0.1% INT and 0.1-0.2 mg membrane protein, and was incubated for up to 45 min at 37°C in sealed test-tubes, and in both the presence and absence of 0.1% (w/v) saponin. The reaction volume was 1 ml. All solutions were preincubated for 5 min at 37°C prior to the start of the assay. A stock solution of 0.2% INT in 100mM-K_2HPO_4/KH_2PO_4, pH 7.4, was solubilized prior to the assay by warming for 3 hr at 37°C. The reaction was started by the

addition of the INT and stopped by adding 1 ml 10% TCA, and the formazan produced (the reduced form of INT which is insoluble in water) was solubilized by adding 4 ml ethyl acetate. The tubes were shaken vigorously to ensure the complete solubilization of the formazan, and then left for 30 min to allow the separation of the two phases. Samples of the ethyl acetate extract containing the formazan were clarified by the addition of a tenth of a volume of methanol (Michell *et al.*, 1970) and their absorbances measured at 490 nm (the molar extinction coefficient is 20100 cm^2 mol^{-1} at 490 nm).

2.2.1.3.6 NADPH-cytochrome c reductase

The activity of NADPH-cytochrome c reductase was measured spectrophotometrically at 37°C in a Philips PU 8700 series spectrophotometer by following the reduction of cytochrome c at 550 nm (Sottocasa *et al.*, 1967). The assay mixture consisted of 6 mM-K_2HPO_4/KH_2PO_4, pH 7.4, 3 mM-$MgCl_2$, 3 mM-ADP, 130 mM-sucrose, 0.12 mM-NADPH, 0.032 mM-cytochrome c and 0.1-0.2 mg membrane protein, and was incubated in both the presence and absence of 0.1% (w/v) saponin. The reaction was started by the addition of the cytochrome c and the reaction volume was 1 ml. All solutions were preincubated for 5 min at 37°C prior to the start of the assay. The activity of the enzyme was expressed in units of change in absorbance per min per mg membrane protein.

2.2.1.4 Electron microscopy

Samples were prepared for ultrathin sectioning as follows. An aliquot of each membrane fraction (containing about 1 mg protein) was pelleted by centrifugation at 100,000g (Beckman 70.1Ti rotor, r_{av}. 6.1 cm). Each pellet was then fixed for 1 hr in the first fixative, which consisted of 0.1 M-sodium cacodylate, pH 7.4, 2% paraformaldehyde, 2.5% glutaraldehyde and 2 mM-$CaCl_2$. All procedures were carried out at room temperature and in a fume cupboard. The pellets were washed in three changes of 0.1 M-sodium cacodylate, pH 7.4, 2% paraformaldehyde, 2.5% glutaraldehyde, 2 mM-$CaCl_2$ for a total of 2 hr and finally fixed again for 1 hr in the second fixative, which consisted of 0.1 M-sodium cacodylate, pH 7.4, 1% osmium tetroxide and 2 mM-$CaCl_2$. The samples were then dehydrated by placing in the following solutions in turn; 50%, 70%, 80%, 90%, and 95% ethanol, each for 10 min, followed by three changes of 100% ethanol and two changes of propylene oxide, each for 15 min. Finally, the samples were embedded by the following procedure. The specimens were infiltrated in glass vials with 50% propylene oxide and 50% araldite mixture (the araldite mixture consisted of 49% araldite CY212, 49% dodecenyl succinic anhydride, and 2% benzyldimethylamine) for 30 min on a rotating holder and then transferred to 100% araldite mixture and infiltrated in uncapped vials overnight on the rotator. The next day the samples were infiltrated with fresh araldite mixture for a further 6 hr on the rotator before being transferred to dry embedding moulds, filled with araldite mixture and polymerized for 48 hr at 60°C.

The polymerized blocks were sectioned, and the thin sections stained and examined in a Jeol 100S transmission electron microscope by Colin MacFarlane of the Botany Department, University of Edinburgh.

2.2.2 ADP-ribosylation assay

2.2.2.1 General method

The ADP-ribosylation assay mixture consisted of about 50 μg membrane protein incubated with 20 μg/ml activated toxin (see section 2.2.2.2), 5 mM-ATP, 0.5 mM-GTP, 200 mM-K_2HPO_4/NaH_2PO_4 buffer, pH 7.5, 10 mM-creatine phosphate, 50 U/ml-creatine phosphokinase, 10 mM-thymidine, 10 mM-isoniazid, 10 mM-$MgCl_2$, 2 mM-EDTA and 5 μM-[adenylate-^{32}P]NAD^+ (25-35 Ci/mmol). Thymidine was added to inhibit poly ADP-ribosylation, isoniazid to inhibit NAD^+-glycohydrolase activity, and creatine phosphate and creatine phosphokinase were added as an ATP regenerating system. The final reaction volume was 100 μl. All solutions were preincubated for 5 min at 30°C and the reaction was started by the addition of the membranes. The reaction mixture was incubated for 30 min at 30°C and the reaction stopped by dilution with 1 ml of ice-cold 10 mM-Mops, pH 7.5, 0.13 M-NaCl. The reaction mixture was then centrifuged at 9,000g (MSE Micro Centaur) for 20 min and the supernatant removed carefully using a syringe. Pellets were solubilized by boiling for 20 min in 60 μl 0.14 M-Tris/HCl, pH 8.8, 25% (v/v) glycerol, 10% (w/v) SDS, 30 mM-dithiothreitol, 0.01% (w/v) bromophenol blue, and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% gel (see section

2.2.2.3). The gel was stained for protein, dried and autoradiographed using Agfa-Gevaert x-ray film (see section 2.2.2.4). The incorporation of ^{32}P into membrane proteins was estimated by densitometric analysis (see section 2.2.2.5).

2.2.2.2 Activation of cholera toxin

Cholera toxin was activated prior to use in the ADP-ribosylation assay. This was to ensure the complete separation of the A_1 from the A_2 chain. The activation buffer used consisted of 10 mM-Mops, pH 7.5, 7 mM-dithiothreitol, 0.13 M-NaCl, 0.01% (w/v) SDS, 1% (w/v) BSA, and 190 µl of this buffer was incubated with 10 µl 10 mg/ml cholera toxin for 30 min at 37°C. This gave a final cholera toxin concentration of 500 µg/ml.

2.2.2.3 Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was routinely carried out under denaturing conditions by the addition of 0.1% (w/v) SDS (SDS-PAGE) to both the stacking and the separating gels. The gel and buffer system used was based on that of Laemmli (1970) with the addition of 2 mM-EDTA to chelate any metal ions which would interfere with the polymerization of the acrylamide and cause the aggregation of proteins (Douglas and Butow, 1976). Polyacrylamide of molecular mass up to $5x10^6$ Da was added to 0.4% (w/v) in the separating gel to increase the mechanical strength. Protein samples were applied to the gel and electrophoresed at 100 V for one hour, while the proteins moved into the stacking gel, and

then at 60 V overnight. Gels were fixed in 20% (v/v) methanol, 10% (v/v) acetic acid for 15 min, stained in 0.25% (w/v) coomassie brilliant blue R, 45% (v/v) methanol, 9% (v/v) acetic acid for 15 min at 55°C, and destained in 5% methanol, 7.5% acetic acid (v/v) at 55°C until the gel background was clear. The addition of pieces of polystyrene foam quickened the destaining process.

For the determination of the molecular masses of membrane proteins, gels were calibrated with the following standard proteins: bovine milk α-lactalbumin (14.2 kDa), soybean trypsin-inhibitor (20.1 kDa), bovine pancreas trypsinogen (24 kDa), bovine erythrocyte carbonic anhydrase (29 kDa), rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (36 kDa), egg albumin (ovalbumin) (45 kDa), bovine serum albumin (66 kDa), rabbit muscle phosphorylase b (subunit) (97.4 kDa), *E. coli* β-galactosidase (subunit) (116 kDa) and rabbit muscle myosin (subunit) (205 kDa). These proteins were supplied as standard kits from Sigma Chemical Co.: Dalton Mark VII-L™ molecular weight marker kit for the determination of molecular masses in the range 14.2 to 66 kDa and a high molecular weight standard mixture for the determination of molecular masses in the range 29 to 205 kDa.

2.2.2.4 Autoradiography

After destaining, the gels were dried using a Bio-Rad model 443 slab gel dryer onto Whatman 3mm filter paper under vacuum for 1 hr at 80°C. Before drying, gels were covered with Saran Wrap™, a nonporous plastic film. Once dried, the Saran Wrap™ was removed and the gels were exposed to Agfa-Gevaert x-ray film in a Du Pont Cronex cassette containing 'lightning plus' intensifying screens and left at -70°C for 2-7 days. Gels were routinely exposed to film for short and long periods: short periods so that the x-ray film was not saturated and so could be analyzed densitometrically, and long periods to see if there were any weakly labelled proteins. Films were developed automatically using a Fuji RGII x-ray film processor.

2.2.2.5 Densitometric analysis

Both the gels and the autoradiographs were scanned using a Joyce Loebl Chromoscan 3 densitometer linked to a DCS turbo (IBM PC clone) computer, keyboard, monitor and Epson LX-80 dot matrix printer. The software used to analyze the data was a Joyce Loebl Chromoscan 3 External Data Analysis Package. Gels and autoradiographs were scanned using a 530 nm filter and absorbance signals corresponding to the amount of protein on gels and of radiolabel on autoradiographs were converted to arbitrary units from the printout of integrated values. In a particular experiment the same amount of protein was present in each assay tube and so the same amount of protein would be expected to be loaded in each well of the gel. To check that this was the case, all gel

tracks were scanned densitometrically after staining and if any of the integrated values obtained for these tracks were greater than 5% different from each other, then the corresponding integrated values for the radiolabel on the autoradiographs were normalized to account for this. The proportionality of densitometry (i.e. the linear range) was determined by varying the exposure times and the amounts of radioactivity exposed to x-ray film.

2.2.3 Adenylate cyclase assay

2.2.3.1 General method

Adenylate cyclase was assayed by a method based on that of Salomon (1979) by determining the amount of cyclic [^{32}P]AMP produced from [α-^{32}P]ATP. The reaction mixture consisted of 40 μl of assay cocktail, and either (i) 35 μl membrane protein (containing 50 μg protein) in 20 mM-Mops, pH 7.5, and 25 μl 40 mM-sodium fluoride, or (ii) 60 μl membranes (containing 50 μg protein) ADP-ribosylated by cholera toxin as described in section 2.2.2.1 except that the [adenylate-^{32}P]NAD$^+$ was replaced with 20 mM-NAD$^+$, giving a total reaction volume of 100 μl. The assay cocktail consisted of 125 mM-Mops, pH 7.5, 2 mM-ATP, 25 μM-GTP, 30 mM-creatine phosphate, 125 U/ml-creatine phosphokinase, 6.25 mM-cyclic AMP, 2.5 mM-dithiothreitol, 25 mM-$MgCl_2$, 2.5 U/ml-adenosine deaminase, 0.25 mM-isobutylmethylxanthine (IBMX), 0.25 mg/ml BSA and 5-15x10^7 cpm/ml [α-^{32}P]ATP. Adenosine deaminase was added to deaminate any adenosine which might be present, thus preventing it from inactivating adenylate cyclase: adenosine activates the inhibitory regulatory subunit of adenylate cyclase. IBMX was added as a phosphodiesterase inhibitor. All solutions were preincubated for 5 min at 30°C and the reaction was started by the addition of the

membranes. The membranes that were used for investigating the effect of sodium fluoride on adenylate cyclase activity were preincubated for 30 min before using in the adenylate cyclase assay. This was to ensure that they had been incubated for the same length of time as those membranes that were ADP-ribosylated by cholera toxin. The reaction mixture was incubated for 20 min at 30°C, and the reaction stopped by adding 25 µl 0.5 M-HCl containing about 10,000 cpm cyclic [8-^{3}H]AMP (to determine the recovery in the later chromatographic step). The tubes were boiled for 2 min, and immediately placed on ice for 10 min. After neutralizing the samples with 25 µl 1.0 M-imidazole (the pH of the reaction mixture after the addition of the 0.5 M-HCl was about 1.4), 1 ml 0.1 M-imidazole/HCl buffer, pH 7.3, was added and the samples passed through chromatographic columns containing 1 g prewashed alumina (see section 2.2.3.2) according to the method of White (1974), in order to separate the cyclic [^{32}P]AMP from the unreacted [^{32}P]ATP. The highly charged unreacted [α-^{32}P]ATP binds to the alumina, whereas the newly formed cyclic[^{32}P]AMP passes through the columns on the addition of buffer. After washing the columns with 1 ml 0.1 M-imidazole/HCl buffer, pH 7.3, and allowing the eluate to completely drain, a further 2 ml was added and the eluate collected (see section 2.2.3.3) and counted in 14 ml scintillation fluid (see section 2.2.3.4). Recoveries from the columns were 65-75% and were calculated by determining the difference between the number of counts per minute of cyclic [8-^{3}H]AMP loaded onto the column and the number of counts per minute eluted.

2.2.3.2 Packing alumina columns

Disposable polystyrene columns (from Pierce) were partially filled with 0.1 M-imidazole/HCl buffer, pH 7.3, and 1 g dry neutral alumina was added such that it fell through the buffer without trapping any air. When the alumina columns were prepared it was found that the pH of the buffer that was initially eluted was higher than that of the buffer itself. Therefore, the columns were washed with buffer until the pH of the eluate was the same as that of the buffer. The volume of wash buffer required was found to be 15 ml. The columns were stored filled with 3 ml buffer and washed with 15 ml buffer prior to use. Each column was used only once before being washed out and refilled.

2.2.3.3 Determination of elution volumes for ATP and cyclic AMP

Assay tubes were prepared containing about 4×10^6 cpm/tube [α-^{32}P]ATP and the volume made up to 100 μl with double-distilled water. 25 μl stopping solution (0.5 M-HCl/10,000 cpm cyclic [8-^{3}H]AMP) was added, followed by 25 μl 1 M-imidazole and 1 ml 0.1 M-imidazole/HCl buffer, pH 7.3. The tubes were decanted into the columns and the eluate allowed to drain completely. 1 ml 0.1 M-imidazole/HCl buffer, pH 7.3, was then added to each of the columns and the eluate collected in vials containing 10 ml scintillation fluid and counted (see section 2.2.3.4). This last step was repeated four times. It was found that all of the ATP was retained in the columns, whereas 65-75% of the cyclic AMP was eluted by the combined second and third 1 ml of buffer. Therefore,

it was decided that after decanting the reaction mixtures into the columns, the columns should be washed with 1 ml of buffer and allowed to drain, and then a further 2 ml of buffer should be added and the eluate collected and counted.

2.2.3.4 Liquid scintillation counting

All radioactive samples produced in the adenylate cyclase assay were counted in 14 ml LKB Optiphase Safe scintillation fluid. All samples were identical with respect to volumes, etc., and for each experiment background and standard vials were also counted. The background vial consisted of 14 ml scintillation fluid and 2 ml 0.1 M-imidazole/HCl buffer, pH 7.3. Two standard vials were counted: the ^{32}P standard vial consisted of 14 ml scintillation fluid, 2 ml 0.1 M-imidazole/HCl buffer, pH 7.3, and 40 µl of a 1:100 dilution of the assay cocktail; and the ^{3}H standard vial consisted of 14 ml scintillation fluid, 2 ml 0.1 M-imidazole/HCl buffer, pH 7.3, and 25 µl of the stopping solution (0.5 M-HCl/10,000 cpm cyclic[8-^{3}H]AMP).

2.2.4 Phosphorylation assay

The phosphorylation assay mixture consisted of about 50 µg membrane protein incubated with 20 µg/ml activated toxin (see section 2.2.2.2), 0.5 mM-GTP, 200 mM-K_2HPO_4/NaH_2PO_4 buffer, pH 7.5, 10 mM-thymidine, 10 mM-isoniazid, 10 mM-$MgCl_2$, 2 mM-EDTA, 20 mM-NAD^+, 50 mM-Mops, pH 7.5, 1 mM-dithiothreitol, 0.1 mg/ml BSA, 0.1 mM-IBMX, 1 U/ml-adenosine deaminase, 0.1% (w/v) saponin and 10 µM-[γ-^{32}P]ATP (10-15 Ci/mmol). The final reaction volume was 100 µl. All

solutions were preincubated for 5 min at 30°C and the reaction was started by the addition of the membranes. The reaction mixture was incubated for 1 min at 30°C and the reaction stopped by adding 1 ml 15% trichloroacetic acid / 45% ethanol. After leaving on ice for 10 min the reaction mixture was centrifuged at 9,000g (MSE Micro Centaur) for 5 min and the supernatant removed. Pellets were solubilized by boiling for 30 min in 60 µl 0.14 M-Tris/HCl, pH 8.8, 25% (v/v) glycerol, 30 mM-dithiothreitol, 10% (w/v) SDS, 0.01% (w/v) bromophenol blue, and analyzed by SDS-PAGE on a 10% gel (see section 2.2.2.3). The gel was stained for protein, dried and autoradiographed using Agfa-Gevaert x-ray film (see section 2.2.2.4). The incorporation of ^{32}P into membrane proteins was estimated by densitometric analysis (see section 2.2.2.5).

CHAPTER THREE

PREPARATION OF MEMBRANES

3.1 Introduction

In order to investigate any differences in the biochemical processes occurring on the luminal (brush border) and contraluminal (basal and lateral) sides of the intestinal epithelial cell it was necessary to separate the brush border membrane from the basal-lateral membrane. There are many methods available for the purification of membrane fractions, including density gradients (Douglas *et al.*, 1972; Mircheff and Wright, 1976; Murer *et al.*, 1976; Yakymyshyn *et al.*, 1982) and divalent cation precipitation techniques (Schmitz *et al.*, 1973; Christiansen and Carlsen, 1981; Stieger and Murer, 1983; Aubry *et al.*, 1986). The problem with these methods is that they generally lead to the purification of only one or the other of the membrane fractions, and not of both at the same time. Generally, when preparing membranes either (i) a crude plasma membrane fraction is produced from an homogenate of the cells which is then subjected to two different procedures to yield the final purified fractions (for example see Colas and Maroux, 1980) or (ii) the final purified membranes are produced completely independently by different methods, but from the same homogenized cells (for example see Domínguez *et al.*, 1985, 1987; Lazo *et al.*, 1985); in both cases the basal-lateral membranes might be prepared by using a sorbitol or sucrose gradient, whereas the brush border membranes might be prepared by cation precipitation. Different procedures employed in the isolation of luminal and contraluminal plasma membranes make it difficult to evaluate whether the enzyme has been preferentially inactivated by one of them,

even if the length of the isolation procedure is the same. This is obviously disadvantageous since it is then hard to compare activities of marker enzymes and so the results of any biochemical experiments on the two fractions will be hard to interpret. Due to this difficulty it appears that many workers have concentrated on the preparation and characterization of either basal-lateral membranes (Douglas *et al.*, 1972; Mircheff *et al.*, 1979) or brush border membranes (Schmitz *et al.*, 1973; Kessler *et al.*, 1978; Hauser *et al.*, 1980; Stieger and Murer, 1983).

Another problem with these methods is the time taken in the purification procedures. Many workers use procedures for preparing say, basal-lateral membranes by density gradient centrifigation taking up to 24 hr, and yet produce brush border membranes by a different procedure taking only a couple of hours. This means that there will be considerably more inactivation of enzymes in one purified fraction compared to the other, again making comparisons of enzymic activities meaningless. Some workers have tried to get around this problem by using differential centrifugation to produce two pellets, one containing brush border membranes and one containing basal-lateral membranes, and then purifying them by using the same density gradient procedure for both (Mircheff and Wright, 1976). However, the 24 hr spin used by Mircheff and Wright (1976) may still result in the inactivation of enzymes particularly the enzyme of interest, adenylate cyclase, which is very labile (Parkinson *et al.*, 1972).

The technique of cation precipitation, introduced by Schmitz *et al.* (1973), is a simple and rapid technique which is commonly used by many workers. However, this procedure seems to be good only for preparing purified brush border membranes (Schmitz *et al.*, 1973; Hauser *et al.*, 1980; Christiansen and Carlsen, 1981; Stieger and Murer, 1983; Aubry *et al.*, 1986). The reason for this appears to be that the brush border membranes are prepared from a crude plasma membrane preparation also containing basal-lateral membranes, mitochondria, nuclei, Golgi apparatus membranes and microsomes. When the divalent cation is added all the membranes, except the brush border membrane fragments, aggregate into large particles which can be sedimented at a low gravitational force: the aggregation is probably due to electrostatic interactions between the cation and membrane surface charges. The failure of brush border membranes to aggregate probably indicates that, despite the negatively-charged glycocalyx (Ito, 1969), the brush border membrane surface, at or near neutrality, is more positively charged than the microsomal, mitochondrial and basal-lateral membrane surfaces. This means that although the brush border fraction is relatively pure, the basal-lateral fraction is contaminated with fragments of cell organelles. This problem could be resolved by subjecting the membranes to a series of centrifugation steps to remove any contaminating membranes before precipitating with the cation. The three methods of divalent cation precipitation that have been used are (i) Ca^{2+}, (ii) Mg^{2+}, and (iii) Mg^{2+}/EGTA. There have been many diverse reports on which is

the best method for precipitating membranes. Aubry *et al.* (1986) compared the procedures involving Ca^{2+} and Mg^{2+} precipitation by analyzing the marker enzyme content and lipid composition of the membranes to establish which method yielded the purest and most structurally intact fractions. They came to the conclusion that Ca^{2+} prepared membranes were less contaminated by basal-lateral membranes than were Mg^{2+} prepared membranes and that the use of Ca^{2+} did not notably enhance the degradation of endogenous lipids by brush-border membrane phospholipase A. Hauser *et al.* (1980) recommended using EGTA and Mg^{2+} instead of Ca^{2+} to aggregate contaminating membranes. They found that membranes prepared using Ca^{2+} had exceptionally high levels of lipid decomposition, and that this was dramatically reduced by using EGTA to keep the free Ca^{2+} concentration low so that the intrinsic Ca^{2+}-activated phospholipase A was inactivated. Stieger and Murer (1983), however, have found that brush border membrane vesicles prepared by the Mg^{2+}/EGTA method are not homogeneous. This could be because (i) the Mg^{2+} precipitation step does not remove all the basal-lateral membranes, (ii) vesicles are formed during the homogenization, which consist of both brush border and basal-lateral elements, or (iii) since the mucosa is scraped with a glass slide there may be cells of different types and stages of differentiation present during the homogenization: membranes of undifferentiated cells have different enzymic patterns to those derived from the tip of the villi (Weiser, 1973).

Thus, although the method of Ca^{2+} precipitation may produce a purer brush border membrane fraction, it may cause the activation of adenylate cyclase or increase the phosphorylation of membrane proteins, rendering it impossible to distinguish the effects due solely to cholera toxin from those due to Ca^{2+}. Therefore, the best cation precipitation method would seem to be that involving the use of Mg^{2+}, as used by Christiansen and Carlsen (1981), rather than Ca^{2+}. Christiansen and Carlsen (1981) estimated that they got a minor contamination of brush border membranes with basal-lateral membranes of approximately 10%.

3.2 Preparation of rat intestinal membrane fractions

3.2.1 Method

Three male Sprague-Dawley rats were sacrificed by cervical dislocation and the mixed plasma, brush border and basal-lateral membrane fractions prepared by following the procedure outlined in section 2.2.1.1 and Figure 2-1.

3.2.2 Marker enzyme assays and determination of protein

Protein was determined as in section 2.2.1.2 according to the method of Bradford (1976). Three marker enzyme assays were carried out to determine the purity of the fractions produced. These were sucrase (see section 2.2.1.3.1), alkaline phosphatase (see section 2.2.1.3.3) and (Na^+-K^+)-ATPase (see section 2.2.1.3.2). All of the marker enzyme assays were carried out as detailed in the indicated sections, except that they were performed only in the

absence of the detergent saponin.

3.2.3 Electron microscopy of membrane fractions

Samples of homogenate, mixed plasma membranes, brush border membranes and basal-lateral membranes were analyzed and compared by electron microscopy as outlined in section 2.2.1.4.

3.2.4 Activation of adenylate cyclase

The activation of adenylate cyclase by sodium fluoride was determined as in section 2.2.3.1. For details of the calculation involved in determining the amount of cyclic AMP produced see Appendix C.

3.2.5 Results and discussion

The results of the marker enzyme assays, shown in Table 3-1, indicate that there was some separation of the membranes. Sucrase, a marker enzyme for the brush border membrane, had an activity 5.1-fold greater in brush border than in basal-lateral membranes; on the other hand alkaline phosphatase activity was 3.4-fold greater in brush borders. This lower value for alkaline phosphatase may reflect the result obtained by other workers (Mircheff and Wright, 1976), which is that the enzyme appears to be located in both membranes, although to a greater extent in brush borders. (Na^+-K^+)ATPase, a marker enzyme for basal-lateral membranes, had an activity that was 2.5-fold greater in basal-lateral membranes than in brush border membranes.

Table 3-1 Distribution of enzyme activities in rat intestinal membrane fractions

H = Homogenate, MPM = Mixed Plasma Membranes, BLM = Basal-Lateral Membranes and BBM = Brush Border Membranes. S.A.* = Specific Activity in μmol.min^{-1}.(mg protein)$^{-1}$ except for (Na^+-K^+)ATPase which is in nmol.min^{-1}.(mg protein)$^{-1}$.
R.S.A.+ = Specific Activity Relative to the mixed plasma membranes. For details of the preparation and the enzyme assays see section 2.2.1. Values represent mean±SD of four membrane preparations.

Fraction	Protein		Sucrase		Alkaline Phosphatase		(Na^+-K^+)ATPase	
	mg	%	S.A.*	R.S.A.+	S.A.	R.S.A.	S.A.	R.S.A.
H	2336±150	100	0.06±0.007	0.3	0.05±0.005	0.2	6.1±2.1	0.7
MPM	194±14	8.3	0.24±0.021	1.0	0.38±0.040	1.0	8.2±2.5	1.0
BLM	99±9	4.2	0.17±0.012	0.7	0.28±0.025	0.7	9.2±2.9	1.1
BBM	23±4	1.0	0.86±0.071	3.7	0.95±0.087	2.5	3.7±1.1	0.5

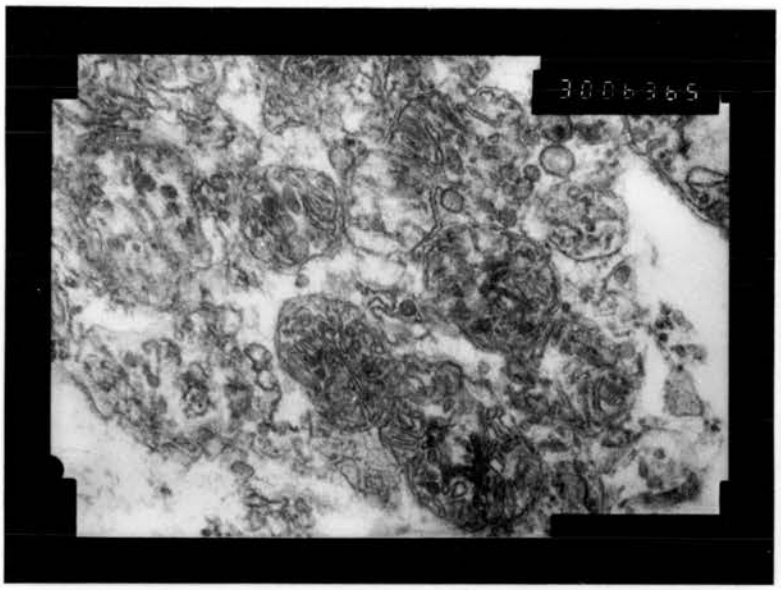
Figure 3-1 (A-E) shows the appearance of whole cells (A and B), mixed plasma membranes (C), brush border membranes (D) and basal-lateral membranes (E) under the electron microscope. The micrographs of the whole cell fraction clearly show recognizable structures, such as mitochondria (A) and nuclei (B), which appear to be absent from the other three fractions. However, the mixed plasma membrane (C) and basal-lateral membrane (E) fractions contain electron-dense material, some of which might be the result of contamination by nuclear or some kind of fibrous material. Although the marker enzyme assays (Table 3-1) show only a relatively small degree of separation compared to other workers (Douglas *et al.*, 1972; Christiansen and Carlsen, 1981; Domínguez *et al.*, 1985), the brush border membrane (D) and basal-lateral membrane (E) fractions do look quite different under the electron microscope; although, both fractions consist of vesicles and membranous sheets. Therefore, prior to improving the separation procedure it was decided to try and assay for adenylate cyclase activity in the mixed plasma, basal-lateral and brush border membrane fractions.

If cholera toxin was used as the activator of the enzyme and the assay yielded no result, it would be difficult to determine whether it was the adenylate cyclase assay or the ADP-ribosylation assay that was responsible: the ADP-ribosylation of the membrane proteins is necessary before they can be used in the adenylate cyclase assay. Therefore, it was decided to use sodium fluoride as the activator since this only involves one assay. However, using sodium

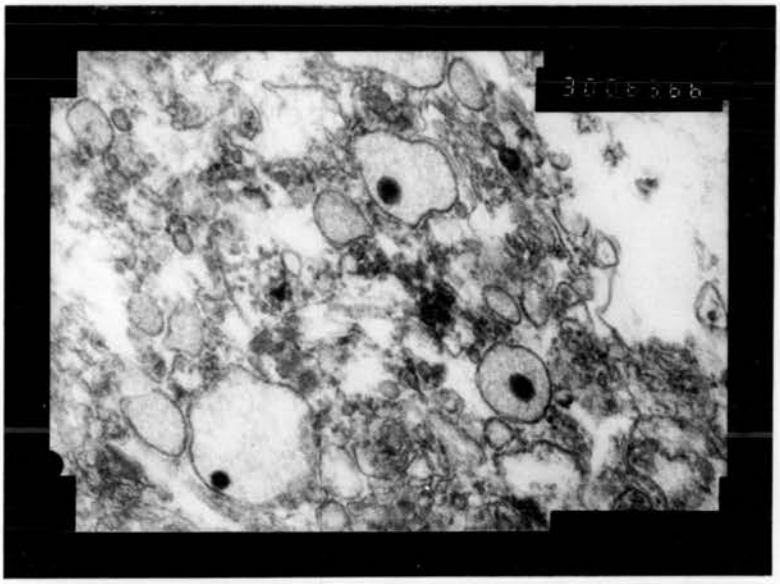
Fig. 3-1 Electron micrographs of rat intestinal membrane fractions

Whole cells (A and B), mixed plasma membranes (C), brush border membranes (D) and basal-lateral membranes (E) were prepared for examination under the electron microscope as outlined in section 2.2.1.4. Magnification x 30,000.

A



B



C

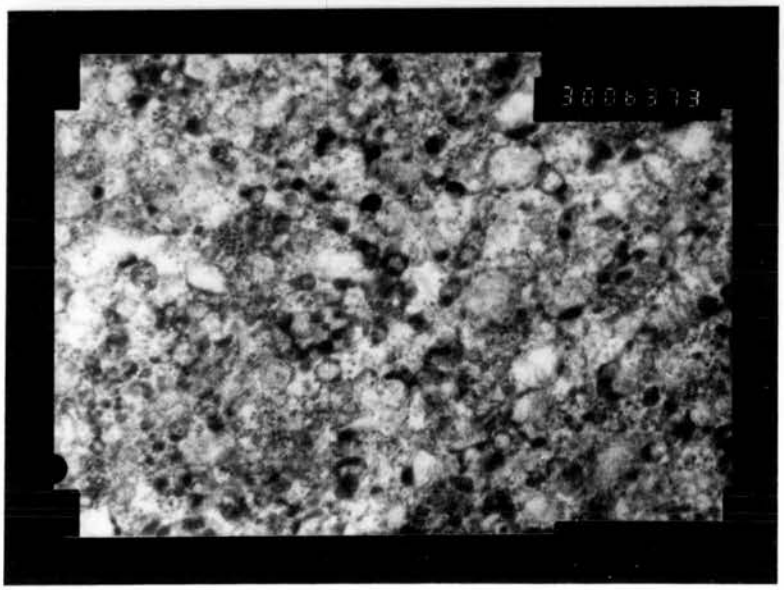
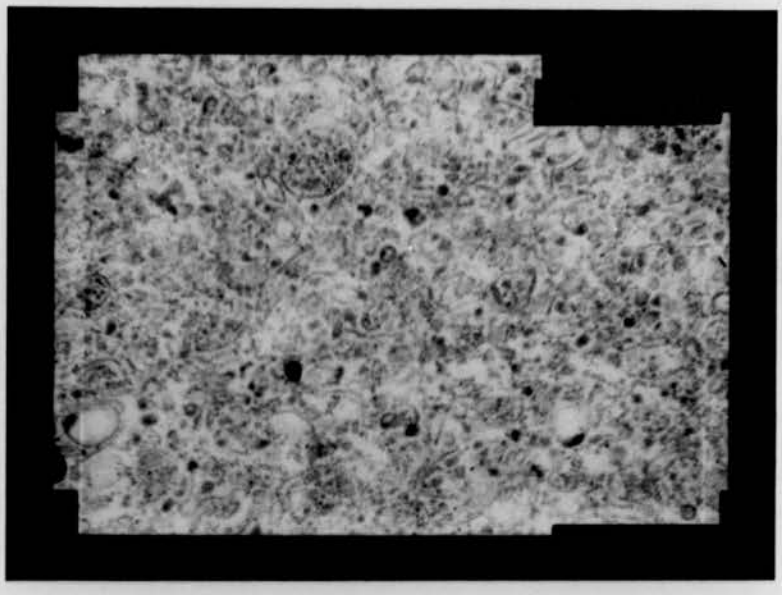


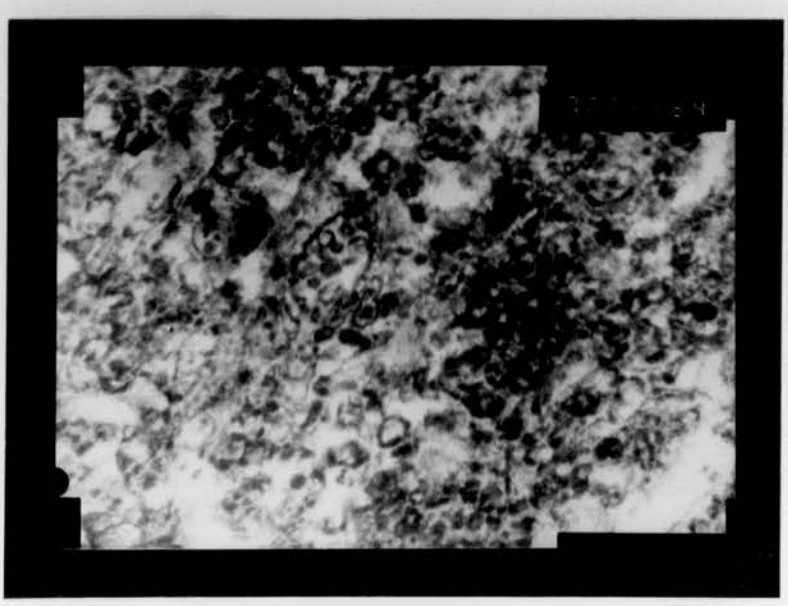
Fig. 3-1 **Electron micrographs of rat intestinal membrane fractions**

Whole cells (A and B), mixed plasma membranes (C), **brush border membranes (D)** and **basal-lateral membranes (E)** were prepared for examination under the electron microscope as outlined in section 2.2.1.4. Magnification x 30,000.

D



E



fluoride, no activation of adenylate cyclase was achieved in any of the three membrane fractions, when compared to the basal values: all values were less than 1 pmol cyclic AMP produced per min per mg membrane protein. This experiment was repeated a number of times and the same result was obtained. This lack of activation could have been due either to the inactivation of the cyclase during the preparation of the membranes, or because of the conditions or the components of the assay itself. In order to discount this latter reason membranes were required that were very rich in adenylate cyclase activity: such membranes were those of pigeon red blood cells (van Heyningen, 1977b). 35 µl 0.3% pigeon red blood cells in 20 mM-Mops, pH 7.5, were incubated with 25 µl 40 mM-sodium fluoride and 40 µl of assay cocktail for 20 min at 30°C as outlined in section 2.2.3.1. The effect of sodium fluoride on adenylate cyclase activity in mixed plasma membranes was also investigated at the same time to compare activities. With the mixed plasma membranes there was still no activation, whereas with the blood cells 384 pmol cyclic AMP was produced per min per ml blood, compared to a basal level of 4 pmol per min per ml. This result therefore implies that the assay conditions were satisfactory and that maybe the cyclase was inactivated during the preparation of the rat intestinal membrane fractions. Other possibilities were that the activity of the cyclase is naturally low in rat intestinal cells and so was easily lost during the membrane preparation, or that maybe the phosphodiesterase activity is higher in these cells. After repeating the assay a few times, changing the

experimental conditions, for example lowering the incubation temperature to 25°C, using a different phosphodiesterase inhibitor (i.e. theophylline), trying fresh membrane preparations, increasing the ATP concentration, and still getting the same result, it was decided to try a different animal model - RABBIT.

3.3 Preparation of rabbit intestinal membrane fractions

3.3.1 Method

One female white New Zealand rabbit was sacrificed by cervical dislocation and the membrane fractions prepared from the intestinal epithelial cells as outlined in section 2.2.1.1 and Figure 2-1.

3.3.2 Marker enzyme assays and determination of protein

Protein was determined as in section 2.2.1.2 according to the method of Bradford (1976). Six marker enzyme assays were carried out to determine the purity of the fractions produced. These were sucrase (see section 2.2.1.3.1), alkaline phosphatase (see section 2.2.1.3.3), (Na^+-K^+)ATPase (see section 2.2.1.3.2), acid phosphatase (see section 2.2.1.3.4), succinate dehydrogenase (see section 2.2.1.3.5) and NADPH-cytochrome c reductase (see section 2.2.1.3.6).

3.3.3 Protein profiles of membrane fractions

The proteins present in each of the membrane fractions (mixed plasma, brush border and basal-lateral) were compared by subjecting 50 μg protein samples to SDS-PAGE on a 10%

gel, as in section 2.2.2.3. The protein samples were solubilized prior to applying to the gel by boiling for 20 min in 60 µl 0.14 M-Tris/HCl, pH 8.8, 25% (v/v) glycerol, 10% (w/v) SDS, 30 mM-dithiothreitol, 0.01% (w/v) bromophenol blue.

3.3.4 Activation of adenylate cyclase

The activation of adenylate cyclase by both cholera toxin and sodium fluoride was determined as in section 2.2.3.1. For details of the calculation involved in determining the amount of cyclic AMP produced see Appendix C.

3.3.5 Results and discussion

The results of the marker enzyme assays are shown in Table 3-2. Several putative plasma membrane enzymes were enriched in the membrane fractions, namely sucrase, alkaline phosphatase and (Na^+-K^+)ATPase, although the (Na^+-K^+)ATPase was not enriched to as great an extent as the other two. About 25% of the total sucrase and 20% of the total alkaline phosphatase present in the homogenate was found in the mixed plasma membrane fraction, whereas only 15% was found in the case of (Na^+-K^+)ATPase. After separating a brush border membrane fraction and a basal-lateral membrane fraction from the mixed plasma membranes by cation precipitation, the activity of sucrase was 18.4-fold greater in the brush border membranes than in the basal-lateral membranes, while alkaline phosphatase had a 8.4-fold greater activity. It is not likely that the increase in activity in basal-lateral membranes and the decrease in activity in brush border

Table 3-2 Distribution of enzyme activities in rabbit intestinal membrane fractions

H = Homogenate, MPM = Mixed Plasma Membranes, BLM = Basal-Lateral Membranes and BBM = Brush Border Membranes. Specific activity is expressed in Units/mg membrane protein (U/mg), where 1 Unit (U) is equivalent to 1 μmol product produced per min, except for NADPH-cytochrome c reductase whose activity is expressed in change in absorbance per min per mg protein. R.S.A.+ = Specific Activity Relative to the mixed plasma membranes. For details of the preparation and the enzyme assays see section 2.2.1. Values represent mean±SD of five membrane preparations.

Fraction	Protein		Sucrase			Alkaline phosphatase			(Na^+-K^+)ATPase		
	mg	%	U/mg	U	R.S.A.+	U/mg	U	R.S.A.	U/mg	U	R.S.A.
H	1284±106	100	0.104±0.009	141±18	0.5	0.065±0.008	89±11	0.6	0.0290±0.009	29±9	0.8
MPM	195±17	15	0.205±0.017	36±5	1.0	0.103±0.009	18±3	1.0	0.0275±0.008	4.2±1.3	1.0
BLM	131±11	10	0.045±0.003	5±0.7	0.2	0.043±0.006	5±0.9	0.4	0.0363±0.010	3.8±1.2	1.3
BBM	39±4	3	0.826±0.071	26±2	4.0	0.360±0.031	12±2	3.5	0.0128±0.004	0.4±0.1	0.5

Fraction	Acid phosphatase			Succinate dehydrogenase			NADPH-cytochrome c reductase	
	U/mg	U	R.S.A.	U/mg	U	R.S.A.	ΔA/min/mg	R.S.A.
H	0.096±0.011	130±16	0.9	0.0568±0.008	62.13±7.23	4.1	0.046±0.010	1.1
MPM	0.111±0.013	20±3	1.0	0.0141±0.002	2.39±0.31	1.0	0.043±0.008	1.0
BLM	0.104±0.009	12±1	0.9	0.0133±0.001	2.04±0.19	0.9	0.094±0.019	2.2
BBM	0.176±0.016	6±0.8	1.6	0.0015±0.001	0.05±0.006	0.1	0.041±0.008	0.9

membranes relative to mixed plasma membranes, for alkaline phosphatase compared to sucrase, is only a result of contamination in the basal-lateral membrane fraction by brush borders. If this was the case, then the same increase in activity in brush borders compared to basal-lateral membranes would be expected for alkaline phosphatase as with sucrase. Instead, this lower enrichment factor is more likely due to alkaline phosphatase being present in both membranes, in agreement with the results of other workers (Mircheff and Wright, 1976). In contrast to the good separation obtained for brush border membranes, as indicated by the 18.4-fold greater activity of sucrase in this membrane fraction compared to the basal-lateral membrane fraction, the activity of (Na^+-K^+)ATPase was only 2.8-fold higher in the basal-lateral membrane fraction than in the brush border membrane fraction. This low enrichment factor could be due to basal-lateral membranes being distributed heterogeneously between the two fractions when the method of Mg^{2+} precipitation is used, as was found by other workers (Stieger and Murer, 1983; Aubry *et al.*, 1986); it could be due to to crosscontamination by membranes other than those from the cell envelopment; it may be due to epithelial cells that have not yet fully differentiated; or it could be a combination of any of these. It should be stressed at this point that the measured ouabain-sensitive K^+-phosphatase activity exhibited by (Na^+-K^+)ATPase gave rather variable results, probably because of the high activity of other phosphatases present in the fractions which may interfere with the assay: all the phosphatase assays were based on the

same method and the greatest phosphatase activity, expressed by alkaline phosphatase, was inhibited by the addition of EDTA to the assay mixture (Hübscher and West, 1965). Generally though, the relative specific activities were the same even if the specific activities differed by a factor of two, although it was the values for the homogenate that were the most variable.

The distribution of mitochondria, lysosomes and smooth endoplasmic reticulum was measured by determining the activities of succinate dehydrogenase, acid phosphatase and NADPH-cytochrome c reductase respectively, in the three fractions. The specific activity of the mitochondrial enzyme was decreased in the mixed plasma membrane fraction compared to the homogenate, while the activities of the lysosomal and smooth endoplasmic reticulum enzymes were unchanged. The reduced mitochondrial activity of the mixed plasma membrane fraction compared to the homogenate was found to be mainly associated with the basal-lateral membrane fraction after precipitation with $MgCl_2$: there was a 8.9-fold greater activity in basal-lateral membranes compared to brush border membranes. Smooth endoplasmic reticulum has historically presented the most difficult problem during the purification of plasma membranes in that it seems to associate preferentially with basal-lateral membranes and is very difficult to remove (Mircheff and Wright, 1976). This accounts for the unchanged specific activity in mixed plasma membranes compared to the homogenate, and the subsequent 2.4-fold increase in specific

activity in basal-lateral membranes compared to brush border membranes. Acid phosphatase, on the other hand, was found to have a 1.7-fold greater specific activity in brush border membranes compared to basal-lateral membranes, as well as the specific activity of mixed plasma membranes being unchanged compared to the homogenate. This is in contrast to other workers (Mircheff and Wright, 1976; Murer *et al.*, 1976) who have found a decrease in specific activity in mixed plasma membranes compared to the homogenate, as well as a preferential association of any contaminating lysosomal membranes with basal-lateral membranes.

As mentioned in Chapter Two, all the marker enzyme assays were performed in both the presence and absence of saponin to ensure that all the enzyme active sites were available for interaction with the substrate, even though it has been reported by Haase *et al.* (1978) that membranes prepared by the method of cation precipitation are obtained as right-side-out orientated vesicles. Kessler *et al.* (1978) reported that sucrase was present on the luminal side of prepared brush border membrane vesicles and suggested that these vesicles were orientated luminal-side-out (i.e. right-side-out). However, some workers (Christiansen and Carlsen, 1981; Yakymyshyn *et al.*, 1982) have found that the majority of the vesicles produced in their brush border membrane preparations were open-ended, thus making it difficult to prove the exclusive luminal-side-out orientation of vesicles. Yakymyshyn *et al.* (1982) found that the activity of alkaline phosphatase increased 3-fold after sonication,

whereas with sucrase no difference was observed. Their conclusion was that this suggested alkaline phosphatase to be located on the inner and outer vesicle surfaces unlike sucrase which was totally confined to the outer membrane surface. These results support the theory that brush border membrane vesicles are exclusively orientated right-side-out, since if this was not the case then sucrase activity would be expected to increase in the same way as that of alkaline phosphatase. In the case of this study no increase in activity was observed with any of the marker enzymes in the presence of saponin, except for alkaline phosphatase, in agreement with Yakymyshyn *et al.* (1982), where the specific activity of each fraction increased 1.5-fold, but still gave the same relative specific activity values. All the values quoted in Table 3-2 are those found in the absence of saponin, although there was not a lot of difference between these values and those found in the presence of saponin.

These marker enzyme assay results therefore show that there has been a good membrane separation as far as brush border membranes are concerned, and that the low specific activity of (Na^+-K^+)ATPase in basal-lateral membranes was probably due to a combination (1) of contamination by intracellular organelles that have not been fully removed prior to the addition of $MgCl_2$ and so are aggregated with the basal-lateral membranes, and (2) of not all the basal-lateral membranes being aggregated by the addition of the $MgCl_2$ in the cation precipitation stage. The protein profiles of each of the three membrane fractions (mixed plasma, brush

border and basal-lateral) on a 10% polyacrylamide gel are shown in Figure 3-2, and show that the brush border and basal-lateral membrane proteins are completely different from each other, whereas the mixed plasma membrane fraction contains proteins from both. Particularly striking are the three protein bands isomaltase, sucrase and actin, which are present to a much greater extent in the brush border membrane fraction and are according to Tiruppathi *et al.* (1986) proteins only present in the brush border membrane. Therefore, having achieved as good a separation of the membrane fractions as other workers who have used the same method of cation precipitation with $MgCl_2$ (Christiansen and Carlsen, 1981; Aubry *et al.*, 1986), or even as those who have used other methods (Murer *et al.*, 1976; Domínguez *et al.*, 1985), it was decided to test these membranes for adenylate cyclase activity.

When sodium fluoride was used as the effector molecule there was an activation of adenylate cyclase in the mixed plasma membrane fraction from a basal level of less than 1 pmol cyclic AMP produced per min per mg membrane protein to 60 pmol per min per mg protein. With cholera toxin the activity of adenylate cyclase was found to be 14 pmol per min per mg protein. Therefore, having achieved a good separation of brush border membranes from basal-lateral membranes, and getting an activation of adenylate cyclase by both cholera toxin and sodium fluoride in these fractions, it was decided to investigate the ADP-ribosylation and phosphorylation of membrane proteins as well as the

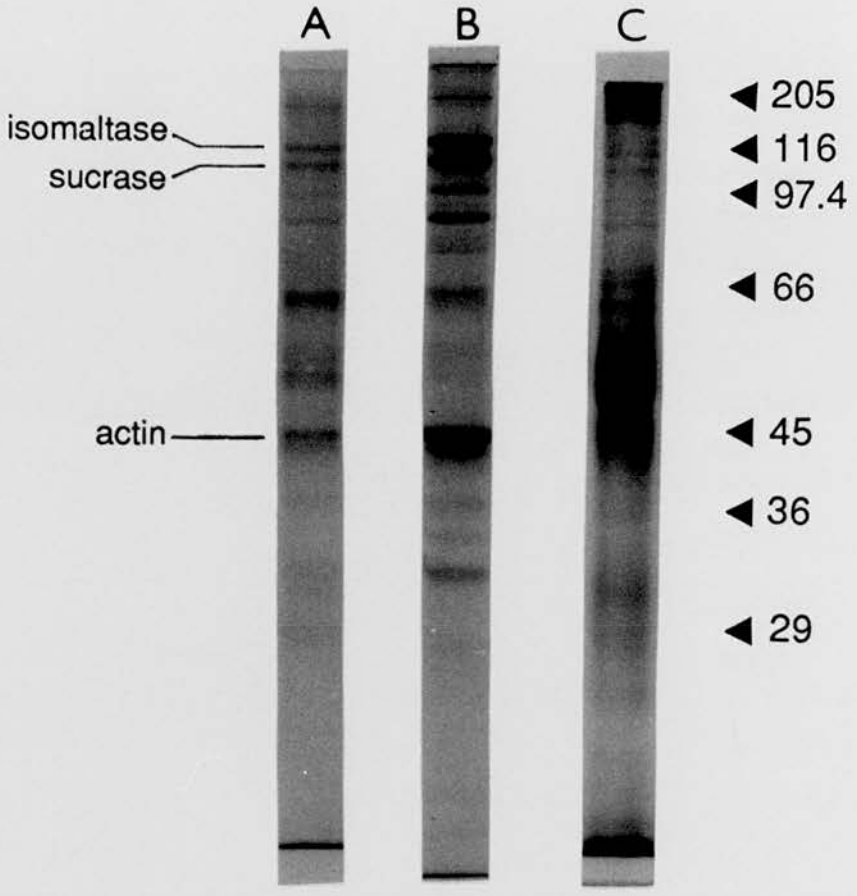


Fig. 3-2 **Protein profiles of the membrane fractions**

A, B and C show the protein profiles of mixed plasma membranes, brush border membranes and basal-lateral membranes respectively on a 10% polyacrylamide gel. The membranes were prepared from rabbit intestinal epithelial cells as outlined in section 2.2.1. The migration of molecular mass standards expressed in kDa is as indicated.

activation of adenylate cyclase in these fractions in detail. The ADP-ribosylation and adenylate cyclase assays described in Chapters Four and Five are based on the same membrane preparation, the marker enzyme assay results of which are shown in Table 3-2, whereas the phosphorylation assay results of Chapter Six are based on a different membrane preparation, but one which has very similar marker enzyme assay results to those shown in Table 3-2.

CHAPTER FOUR

ADP-RIBOSYLATION

4.1 Introduction

After separating the brush border and basal-lateral membranes from a mixed plasma membrane fraction, the ADP-ribosylation of membrane proteins within each of these fractions was investigated. Domínguez *et al.* (1985, 1987) and Lazo *et al.* (1985) showed that cholera toxin catalyzed the ADP-ribosylation of three membrane proteins of molecular mass 40, 45, and 47 kDa in brush border membranes of rabbit intestinal epithelial cells. However, they found no similar effects with basal-lateral membrane proteins and so came to the conclusion that the regulatory component of adenylate cyclase, G_s, was present only in brush border membranes. Although their conclusions appear reasonable, it seems very strange that they did not get any ADP-ribosylation of proteins in the basal-lateral membrane fraction since the marker enzyme results of their membrane purification, which are very similar to results obtained in this study (see Table 3-2), quite clearly show some crosscontamination with brush border membrane proteins. Therefore, in this project, the proteins labelled in each membrane fraction (mixed plasma, brush border and basal-lateral membranes) were initially identified. The incorporation of ^{32}P-ADP-ribose into each membrane protein in each membrane fraction was then determined and compared to the relative specific activities of the marker enzymes.

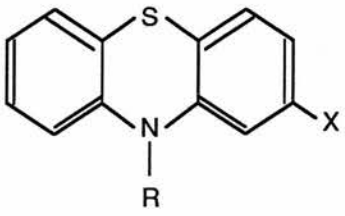
If the G_s protein is located only in the brush border membrane, then the problem is how it gets to the catalytic component of adenylate cyclase, which is reported to be

located on the basal-lateral membrane (Murer *et al.*, 1976; Walling *et al.*, 1978). In 1985 Rodbell proposed a theory which could account for this problem. He depicted the α subunits of the GTP-binding regulatory proteins as being 'programmable messengers' which are released from the plasma membrane when exposed to activating agents such as hormones, F^-, Mg^{2+} or GTP, and bacterial toxins such as cholera toxin. He further proposed that once free these α subunits would be able to interact with other components of the cell and/or plasma membrane. This would then imply that the β and/or γ subunits would behave as moorings for the attachment of the α subunits. Indeed, the work of Sternweis (1986), which shows that the α subunits cannot associate with plasma membranes in the absence of $\beta\gamma$ subunits, supports Rodbell's hypothesis. Evidence has also been provided showing that the ADP-ribosylation of the α subunit of G_s causes its release from rat liver plasma membranes (Lynch *et al.*, 1986). Therefore, in this project an experiment was conducted to try and demonstrate that, upon activation with cholera toxin, there is a release of the major ADP-ribosylated protein from rabbit intestinal brush border membranes.

The effects of six drugs, with differing anticalmodulin and antidiarrhoeal activities, were tested for their ability to inhibit the ADP-ribosylation of membrane proteins: as mentioned in section 1.4.4, the anticalmodulin activities refer to the abilities of the drugs to inhibit the calmodulin-induced activation of phosphodiesterase *in vitro*

(Prozialeck and Weiss, 1982). Five of the drugs tested, namely chlorpromazine (an antipsychotic), trifluoperazine (an antipsychotic), triflupromazine (an antipsychotic), promethazine (an antihistaminic) and promazine (a tranquilizer), belong to the class known as phenothiazines, while the other drug, amitriptyline (an antidepressant), belongs to the class known as dibenzazepines. The structures of these drugs as well as their abilities to inhibit calmodulin activity, expressed as IC_{50} values, are shown in Figure 4-1. These IC_{50} values for the inhibition of calmodulin (Prozialeck and Weiss, 1982; Weiss *et al.*, 1982; Zavecz *et al.*, 1982) (Fig. 4-1) correlate very well with their abilities to inhibit the diarrhoea induced by cholera toxin and other agents, such as prostaglandins and vasoactive intestinal peptide (Ilundain and Naftalin, 1979; Sandhu *et al.*, 1979; Smith and Field, 1980; Lönnroth *et al.*, 1980; Zavecz *et al.*, 1982); chlorpromazine, trifluoperazine and triflupromazine have a greater antidiarrhoeal activity than amitriptyline, promazine and promethazine.

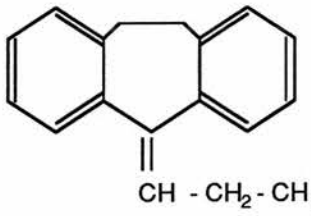
(A)



CLASS :- PHENOTHIAZINES

X	R	Name	IC_{50} (μM)
- Cl	$-CH_2-CH_2-CH_2-N(CH_3)_2$	CHLORPROMAZINE	40
$-CF_3$	$-CH_2-CH_2-CH_2-N(CH_3)_2$	TRIFLUPROMAZINE	28
$-CF_3$	$-CH_2-CH_2-CH_2-N$  $-CH_3$	TRIFLUOPERAZINE	17
- H	$-CH_2-CH_2-CH_2-N(CH_3)_2$	PROMAZINE	110
- H	$-CH_2-CH(CH_3)-N(CH_3)_2$	PROMETHAZINE	340

(B)



CLASS :- DIBENZAZEPINES

Name :- AMITRIPTYLINE IC_{50} :- 100 μM

Fig. 4-1 **Structures and anticalmodulin activities of the antidiarrhoeal drugs**

The six drugs tested belong to the phenothiazine class (A) and the dibenzazepine class (B). IC_{50} values for the inhibition of calmodulin were taken from Prozialeck and Weiss (1982), Weiss *et al.* (1982) and Zavecz *et al.* (1982).

4.2 ADP-ribosylation of membrane proteins

4.2.1 Method

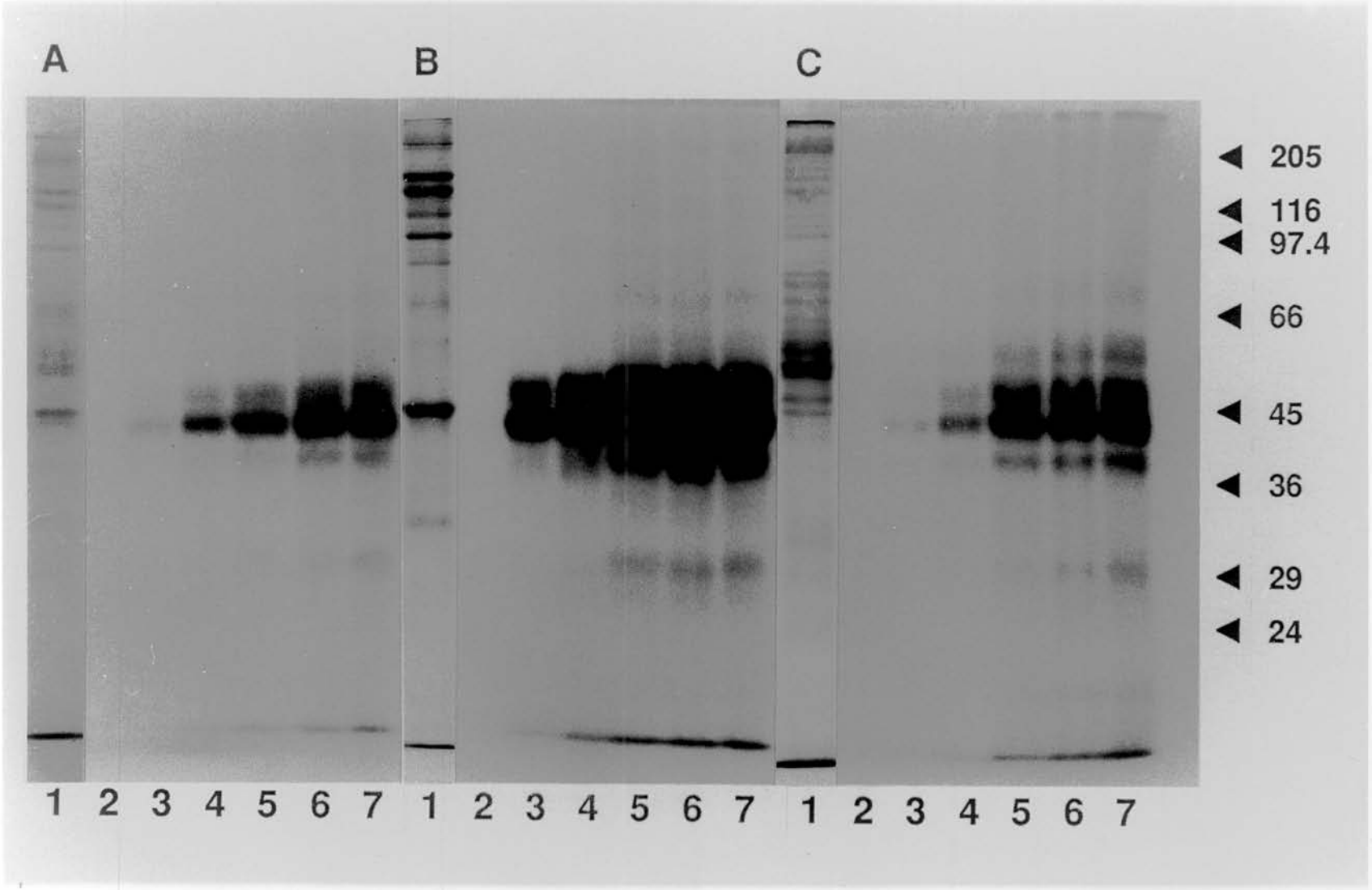
5 μM-[Adenylate-^{32}P]NAD$^+$ and 20 μg/ml preactivated cholera toxin were incubated with 50 μg of either mixed plasma, brush border or basal-lateral membranes at 30°C for 0-60 min, as outlined in section 2.2.2.1, to look at the incorporation of ^{32}P-ADP-ribose into the membrane proteins of each fraction.

4.2.2 Results and discussion

Autoradiographic profiles and graphs from the densitometric analysis of these autoradiographs are shown in Figures 4-2 and 4-3 respectively. Three proteins of molecular mass 45, 40 and 37 kDa from the mixed plasma membrane fraction were labelled (Fig. 4-2A). There were also a number of other weakly labelled proteins (Fig. 4-2). Cholera toxin was also found to catalyze the labelling of these same proteins in both the brush border and basal-lateral membrane fractions (Fig. 4-2B and C). In the absence of cholera toxin no labelling was observed. Figure 4-3 shows that the incorporation of ^{32}P-ADP-ribose, under the conditions of this assay, reached a maximum after about 30 min for each of the three proteins in each of the three fractions. This could be because all the G proteins have been labelled, i.e. fully saturated, or, more likely, because the [adenylate-^{32}P]NAD$^+$ in the assay mixture has been depleted by NAD$^+$-glycohydrolase (also known as NADase). This membrane-bound ectoenzyme is particularly abundant on the surface of macrophages (Artman and Seeley, 1978) and is therefore very

Fig. 4-2 ADP-ribosylation of rabbit intestinal epithelial cell membranes.

A, B and C show autoradiographic profiles of mixed plasma membrane proteins, brush border membrane proteins and basal-lateral membrane proteins respectively, when incubated with 20 μg/ml preactivated cholera toxin and 5 μM-[adenylate-^{32}P]NAD^{+} for 0 min (lane 2), 5 min (lane 3), 15 min (lane 4), 30 min (lane 5), 45 min (lane 6) and 60 min (lane 7) at 30°C as in section 4.2.1. The corresponding protein profiles of mixed plasma membranes, brush border membranes and basal-lateral membranes, which were run on a 10% polyacrylamide gel, are shown in lanes 1A, 1B and 1C respectively (50 μg membrane protein per lane). Migration of molecular mass standards expressed in kDa is as indicated.


A
B
C
205
116
97.4
66
45
36
29
24
1 2 3 4 5 6 7 1 2 3 4 5 6 7 1 2 3 4 5 6 7

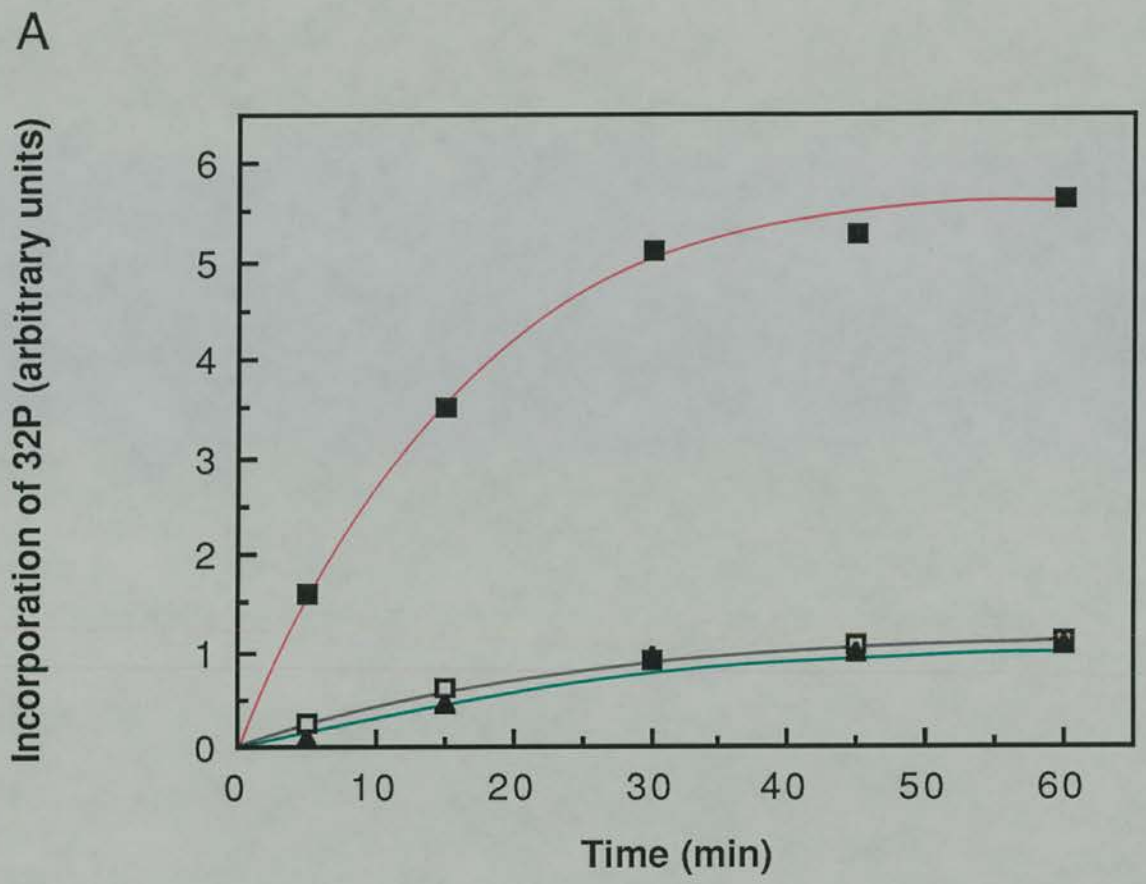
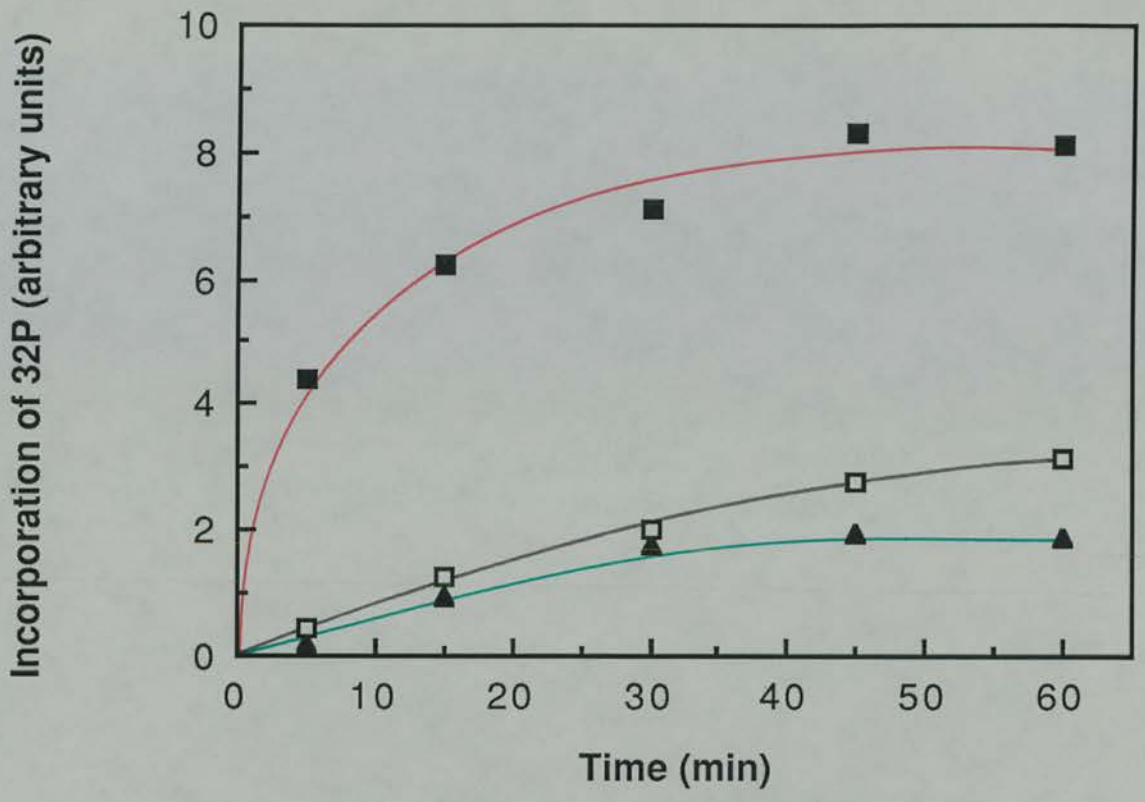


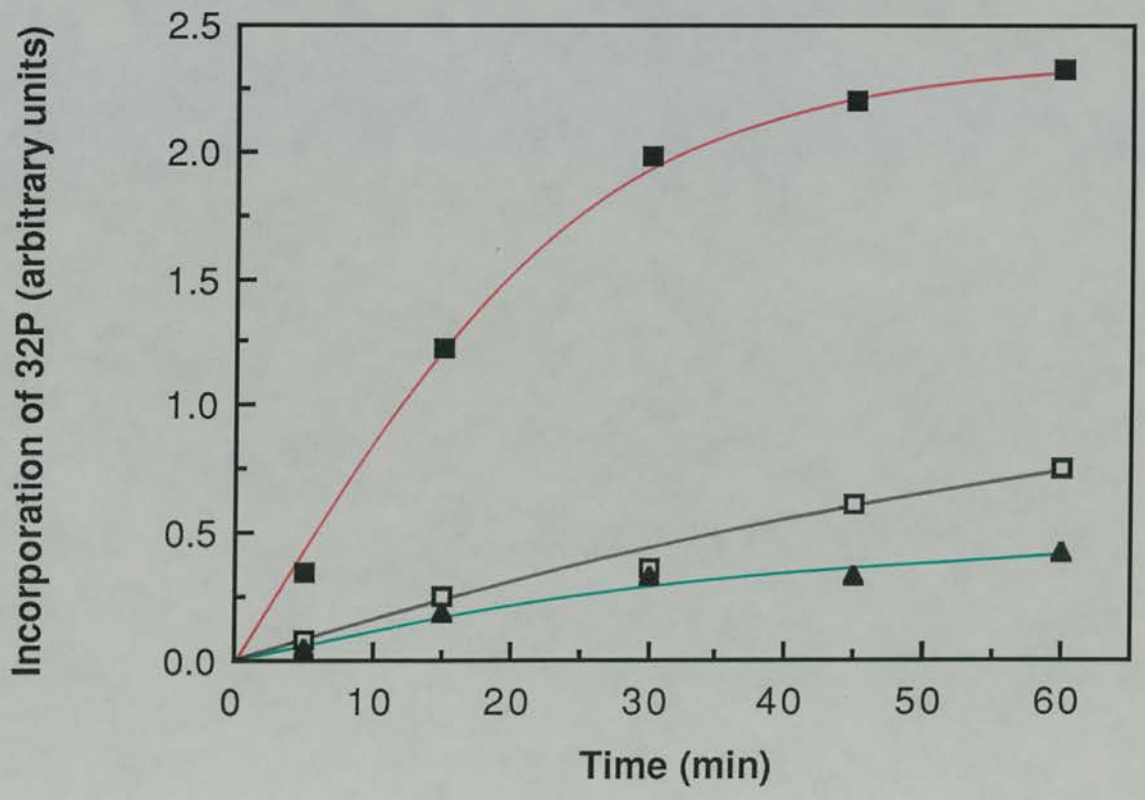
Fig. 4-3 **Time courses for the ADP-ribosylation of membrane proteins.**

Graphs show the incorporation of ^{32}P-ADP-ribose into 45 kDa (A), 40 kDa (B) and 37 kDa (C) proteins of mixed plasma membranes (□—□), brush border membranes (■—■) and basal-lateral membranes (▲—▲). Membranes were incubated with 20 μg/ml preactivated cholera toxin and 5 μM-[adenylate-^{32}P]NAD^{+} at 30°C as in section 4.2.1. The arbitrary units shown were based on the integrated values (arbitrary units x 10^{4}) obtained from the densitometric analysis of the autoradiographs.

B


Incorporation of 32P (arbitrary units)
10
8
6
4
2
0
0
10
20
30
40
50
60
Time (min)

C


Incorporation of 32P (arbitrary units)
2.5
2.0
1.5
1.0
0.5
0.0
0
10
20
30
40
50
60
Time (min)

active in macrophage-rich organs such as spleen, lymph nodes, brain and gut. For a 5% (v/v) suspension of calf brain membranes (2 mg/ml protein) the half life of 10 μM NAD^+ at 37°C has been found to be about 10 s (Gill and Coburn, 1988). This problem of NAD^+ hydrolysis can be alleviated by adding the antituberculous drug isonicotinic acid hydrazide (also known as isoniazid) to inhibit the NADase activity. However, isoniazid does not completely inhibit NADase activity (Kawai *et al.*, 1986; Gill and Coburn, 1988) and so it is inevitable that the [adenylate-^{32}P]NAD^+ will be hydrolyzed before all the G proteins are labelled. To check that this was indeed the case the above experiment was repeated, but for incubation times of 0, 5, 15, 30, 40, 50 and 60 min. After a period of 30 min, extra [adenylate-^{32}P]NAD^+, GTP and ATP (at the same concentrations as at the start of the incubation) was added to those membranes that were being incubated for 40, 50 and 60 min. The results of this experiment, shown in Figure 4-4, show that there was an increase in the incorporation of ^{32}P-ADP-ribose into the 45 and 40 kDa proteins after the addition of the extra substrate and activators. However, there was no increase in the labelling of the 45 kDa protein in the basal-lateral membrane fraction. This must have been due to some experimental artefact, since the labelling of this same protein increased in the mixed plasma and brush border membranes, and so would be expected to increase in the basal-lateral membrane as well. There was no labelling of the 37 kDa protein in this experiment and this was also found to happen occasionally in other experiments; it may be

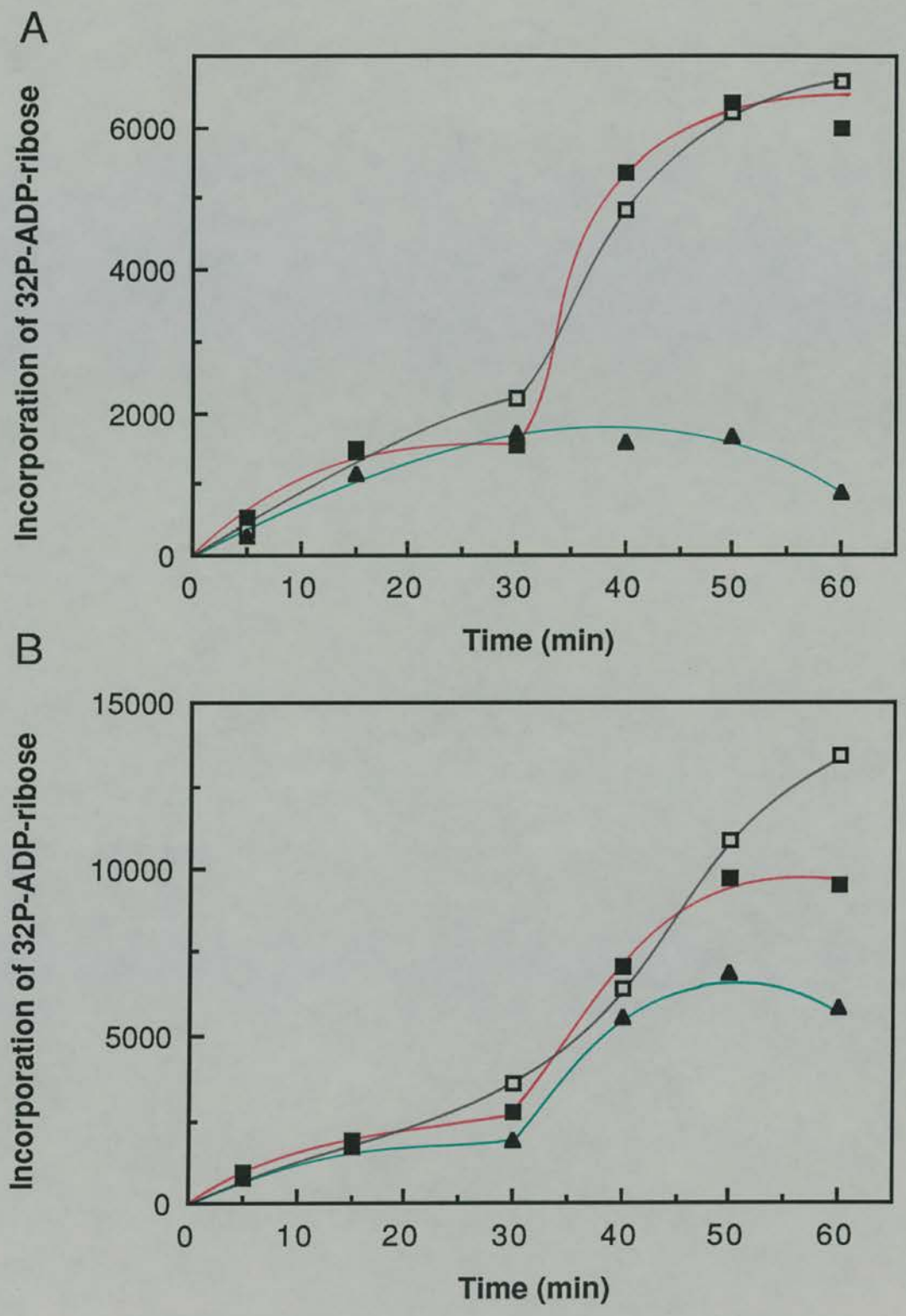


Fig. 4-4 **Effect of adding extra substrate on the ADP-ribosylation of membrane proteins**

Graphs show the incorporation of ^{32}P-ADP-ribose (the units shown are the integrated values from the densitometric analysis of the autoradiographs) into 45 kDa (A) and 40 kDa (B) proteins of mixed plasma membranes (□—□), brush border membranes (■—■) and basal-lateral membranes (▲—▲). Membranes were incubated as in section 4.2.1, and extra [adenylate-^{32}P]NAD$^+$, GTP and ATP were added after incubating for 30 min (see section 4.2.2).

due to a slight alteration in the experimental conditions which caused a reduction in the ADP-ribosylation of this protein, which at its best was quite weak. However, the general trend of an increase in labelling after the addition of more substrate and activators implies that the plateau observed in Figure 4-3 was due to the depletion of the substrate and not the saturation of the G proteins with label.

It is difficult to be certain as to what extent the ADP-ribosylation of proteins is a property of a particular membrane fraction because of membrane crosscontamination. It does not matter how good the membrane isolation procedure is, it is inevitable that during the separation of a particular membrane fraction from a mixture of other cellular organelle membrane fractions there will be some crosscontamination. The labelling of the 45, 40 and 37 kDa proteins in each fraction was compared (Table 4-1) by reading off values from Figure 4-3 corresponding to an incubation time of 5 min: these values represent the integrated values obtained from the densitometric analysis of the autoradiographs shown in Figure 4-2 and drawn graphically in Figure 4-3. The incubation time of 5 min was chosen as the point of reference because this represented a period before the depletion of the substrate, i.e. the linear portion of the time courses shown in Figure 4-3. Table 4-1 shows that the incorporation of ^{32}P-ADP-ribose into the 40 kDa protein of the brush border membrane fraction was 18-fold greater than in the basal-lateral

Table 4-1 Incorporation of ^{32}P-ADP-ribose into membrane proteins

The experimental details of the ADP-ribosylation assay are as in section 4.2.1. Incubation was for 5 min at 30°C. In each of the three membrane fractions row 1 shows the incorporation of ^{32}P-ADP-ribose (integrated values obtained from Figure 4-3), row 2 shows values relative to the labelling of the 40 kDa protein in that membrane and row 3 shows values relative to the labelling of the same protein in the mixed plasma membrane fraction. The relative values represent the mean±SD of three experiments.

Fraction	Row	45 kDa protein	40 kDa protein	37 kDa protein
Mixed	1	2400	4000	900
Plasma	2	0.6±0.06	1.0	0.2±0.04
Membrane	3	1.0	1.0	1.0
Brush	1	16000	45000	4200
Border	2	0.4±0.04	1.0	0.1±0.01
Membrane	3	6.7±0.5	11.3±0.5	4.7±0.3
Basal-	1	1500	2500	600
Lateral	2	0.6±0.02	1.0	0.2±0.01
Membrane	3	0.6±0.04	0.6±0.04	0.7±0.07

membrane fraction. This can be compared with the smaller increase in incorporation for the 45 and 37 kDa proteins of 10.7- and 7-fold respectively, in brush border membranes relative to basal-lateral membranes. The larger incorporation of ^{32}P-ADP-ribose into the 40 kDa protein of brush border membranes relative to mixed plasma membranes after an incubation time of 5 min is responsible for the larger 18-fold greater increase in labelling in brush border membranes compared to basal-lateral membranes. It was a possibility that this larger incorporation of ^{32}P-ADP-ribose in brush border membranes was simply due to an experimental error or artefact, and that the true incorporation was much lower, thus decreasing the value relative to mixed plasma membranes of 11.3 to nearer those for the 45 and 37 kDa proteins of 6.7 and 4.7 respectively. However, the experiment was repeated a further two times and the same initial large increase in labelling in the 40 kDa protein of the brush border membrane fraction was observed, giving an overall profile very similar to that shown in Table 4-1.

These results may be indicative of a small percentage of the 45 kDa protein, which is the same size as the regulatory component of other adenylate cyclases (for a general review see Enomoto and Gill, 1983) being located in the basal-lateral membrane fraction. On the other hand, the major protein labelled in each fraction was the one of 40 kDa (Table 4-1 and Fig. 4-3) and indeed this protein may be $G_{s\alpha}$ since the molecular mass of $G_{s\alpha}$ in intestinal cells has not been determined. If this is the case then the 18-fold greater amount of labelling of this 40 kDa protein in the

brush border as opposed to the basal-lateral membrane fraction is comparable to the enrichment of sucrase in these membranes (about 18.4-fold greater in brush border compared to basal-lateral membranes) (Table 3-2). This would then suggest that the ADP-ribosylation of proteins in the basal-lateral membrane fraction was solely due to contamination with brush border membrane proteins. However, if the 45 kDa protein was $G_{s\alpha}$ then it would seem that the regulatory component is present on both membranes, although to a much greater extent on brush border membranes. Until the identities and relationships of these proteins are known this problem of the location of $G_{s\alpha}$ cannot be resolved (see Chapter Seven).

4.3 Evidence for the release of the α subunit of G_s upon ADP-ribosylation

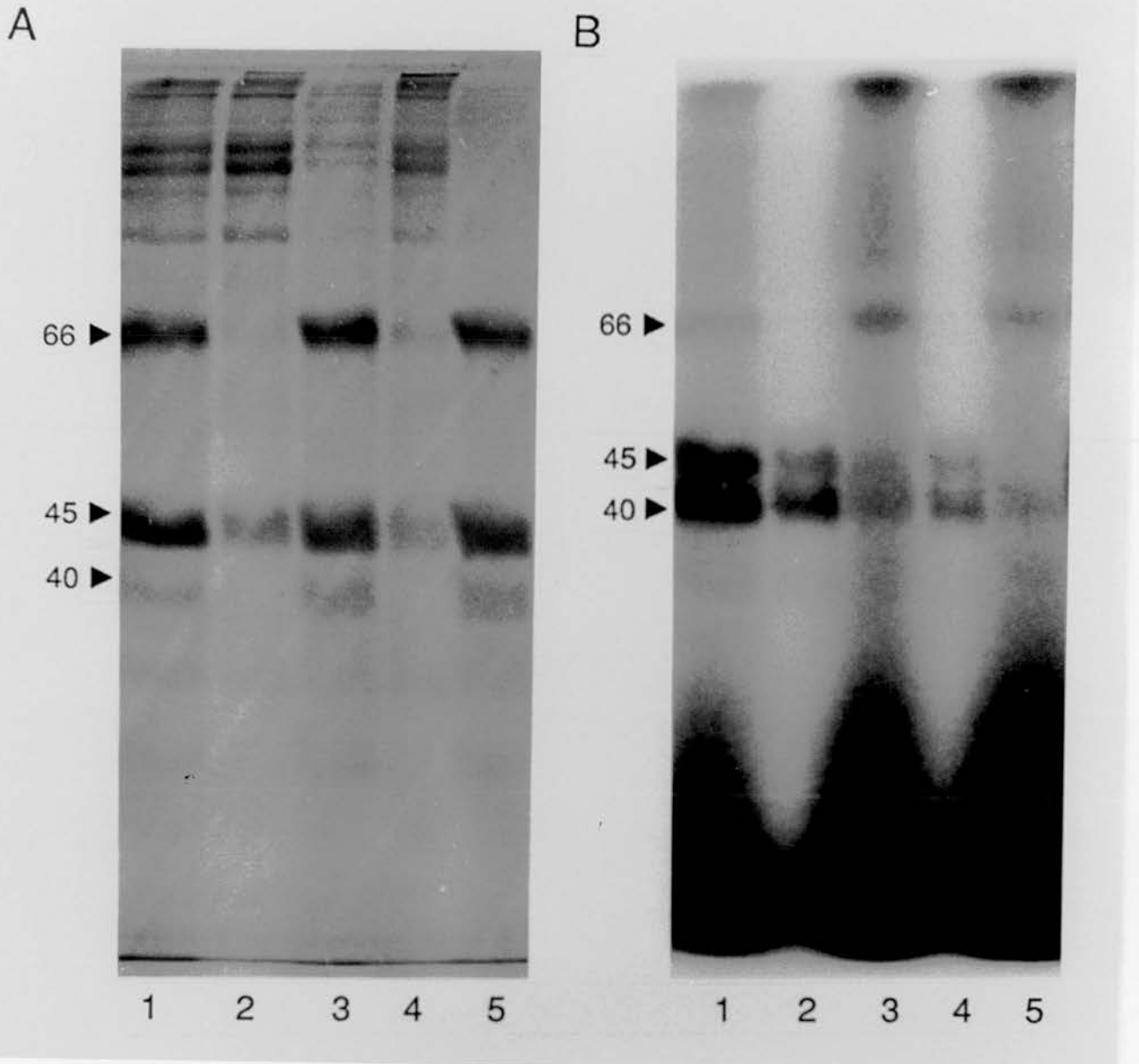
4.3.1 Method

5 μM-[Adenylate-^{32}P]NAD$^+$ and 20 μg/ml cholera toxin were incubated, as in section 2.2.2.1, with 1.5 mg brush border membrane protein in a final reaction volume of 1 ml at 30°C for 20 min. Following this period a 50 μl aliquot was removed (for the determination of total labelling), added to 60 μl 0.14 M-Tris/HCl, pH 8.8, 25% (v/v) glycerol, 10% (w/v) SDS, 30 mM-dithiothreitol, 0.01% (w/v) bromophenol blue (dissociation buffer), and boiled for 20 min. 400 μl of the remaining ADP-ribosylation assay mixture was treated as in section 2.2.2.1 (for the determination of soluble labelled proteins), i.e. transferred to an Eppendorf tube and

centrifuged at 9,000g (r_{av}. 4.62 cm) in a MSE Micro Centaur for 20 min. 50 µl of the resulting supernatant was then boiled for 20 min in 60 µl dissociation buffer, whereas the pellet was resuspended using a syringe in 400 µl of membrane isolation Buffer II (see section 2.2.1.1) and 50 µl of this mixture was then also boiled in 60 µl dissociation buffer for 20 min. To ensure that all the membranes were pelleted by centrifuging the ADP-ribosylation assay sample at 9,000g (MSE Micro Centaur), leaving only soluble proteins in the resulting supernatant, a further 400 µl of the assay mixture was treated as above but with the exception that the membranes were pelleted by centrifuging at 100,000g in a Beckman TL-100 centrifuge (TLA-100.3 rotor, r_{av}. 3.17 cm) for 40 min at 4°C. 50 µl samples, each containing 30 µg protein, of each of the five boiled mixtures were then analyzed by SDS-PAGE (see section 2.2.2.3) on a 10% gel and by autoradiography (see section 2.2.2.4).

4.3.2 Results and discussion

Figure 4-5 shows the gel profile and corresponding autoradiograph of this experiment. The assay was designed such that 30 µg membrane protein was loaded onto each lane of the gel, so that the labelling of proteins in the pellet and supernatant should equal that in the track for total labelling. As far as the gel was concerned, there did not appear to be any difference in the amount of protein loaded in the tracks showing the profiles of the pellet and supernatant resulting from the 9,000g and 100,000g spins. The autoradiograph showed that three proteins of 66, 45 and


A
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2
3
4
5
B
66
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40
1
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5

Fig. 4-5 Release of ^{32}P-ADP-ribosylated proteins from brush border membranes

A and B show the protein profiles, on a 10% polyacrylamide gel, and autoradiographic profiles respectively, when brush border membranes were incubated with 20 μg/ml preactivated cholera toxin and 5 μM-[adenylate-^{32}P]NAD^{+} for 20 min at 30°C as outlined in section 4.3.1. Lane 1 represents total labelling of proteins; lanes 2 and 4 the labelling of membrane proteins found in the pellets resulting from the 9,000g and 100,000g spins respectively; and lanes 3 and 5 the labelling of soluble proteins found in the supernatants also resulting from the 9,000g and 100,000g spins respectively. The molecular masses of the proteins, as indicated, are expressed in kDa.

40 kDa were labelled, but the 37 kDa protein was not. The protein of 66 kDa which was only labelled in the supernatant was the bovine serum albumin present in the cholera toxin activation buffer (see section 2.2.2.2), and which has also been shown to be ADP-ribosylated by Kun *et al.* (1976). The protein was shown to be bovine serum albumin by repeating the ADP-ribosylation assay in the absence of membranes, and running the assay mixture on a 10% gel. The gel and corresponding autoradiograph revealed one band which was labelled and which had a molecular mass of 66 kDa, the same as that of bovine serum albumin. The reason for the high background on the autoradiograph (Fig. 4-5) corresponding to the supernatant and total labelling tracks on the gel, was due to the loading of reaction mixtures containing unreacted ^{32}P-NAD^{+} onto the gel. Both the 45 and 40 kDa proteins appeared to be present in the supernatant tracks as shown by the label on the autoradiograph, although this labelling was quite weak and partially masked by the high background. Table 4-2 shows the integrated values for the labelling resulting from the densitometric analysis of the autoradiograph. The results show that the 9,000g centrifugation step, used in all of the ADP-ribosylation assays, was sufficient to pellet all the membrane-bound proteins. This was shown by the labelling of the 45 and 40 kDa proteins in the pellets, resulting from both the 9,000g and 100,000g spins, being approximately the same. The small differences of 6% and 3% observed in the total labelling (a combination of membrane bound and soluble) of the 45 kDa and 40 kDa proteins respectively, resulting from the 100,000g

Table 4-2 Evidence for the release of the α subunit from the βγ subunits of G_s

Brush border membranes were incubated with 5 μM-[adenylate-^{32}P]NAD$^+$ and 20 μg/ml cholera toxin for 20 min at 30°C as outlined in section 4.3.1. All values in the table are the integral values obtained by the densitometric analysis of the autoradiograph of the 10% polyacrylamide gel (see Fig. 4-5). The percentage values represent the mean±SD of three experiments.

Protein Labelled	Total Labelling	13,000 rpm centrifugation			100,000g centrifugation		
		Pellet~(P)	Supernatant*(S)	(P + S)+	Pellet~(P)	Supernatant*(S)	(P + S)+
66 kDa	5026	0	5695 (113%± 2%)	5695 (113%± 2%)	0	5340 (106%± 3%)	5340 (106%± 3%)
45 kDa	19796	12337 (63%± 5%)	4009 (20%± 2%)	16346 (83%± 7%)	13760 (70%± 6%)	1422 (7%± 3%)	15182 (77%± 9%)
40 kDa	25609	21711 (85%± 7%)	5396 (21%± 3%)	27107 (106%± 10%)	22622 (88%± 5%)	3714 (15%± 3%)	26336 (103%± 8%)

~ The values in brackets represent the values for the pellet as a percentage of the value for total labelling.

* The values in brackets represent the values for the supernatant as a percentage of the value for total labelling.

+ The values in brackets represent the combined values for the pellet and supernatant as a percentage of the value for total labelling.

compared to the 9,000g centrifugation, is probably due to experimental error. Errors in the estimation of the amount of labelling by densitometric analysis may also have arisen due to the high background on the autoradiograph. The experiment was carried out in duplicate and both sets of data gave very similar results. Thus, it appears that upon ADP-ribosylation there was approximately a 20% release of the 40 and 45 kDa membrane-bound proteins into the cytosol. This means that the α subunits of G proteins may be released from the brush border membrane upon ADP-ribosylation by cholera toxin, allowing them to move freely through the cell cytosol to the basal-lateral membrane, at the other side of the cell, where they can interact with the catalytic subunits of adenylate cyclase, which are said to be located only on this membrane (see Chapter Five). Since the $\beta+\gamma$ subunits of G_s have been found to inhibit adenylate cyclase activity (Northup *et al.*, 1983b), then it is safe to assume that the α subunits must be released from these $\beta\gamma$ subunits so that they, the α subunits, can activate adenylate cyclase. Under the normal hormonal regulation of adenylate cyclase it is the binding of GTP to G_s that initiates the activation of the cyclase. It would therefore seem likely that the GTP binds to the α subunits (Northup *et al.*, 1982), causing their release from the $\beta+\gamma$ subunits and allowing them to interact with the catalytic subunit. Therefore, under normal hormonal regulation, the hormone binds its receptor at the basal-lateral membrane, the receptor binds G_s and then GTP binds the α subunit of G_s causing its release from the $\beta+\gamma$ subunits. Maybe the released α

subunits remain associated with the basal-lateral membrane and interact with the catalytic subunits of adenylate cyclase also on this membrane. It then would be possible that cholera toxin ADP-ribosylates GTP-bound α subunits of cholera-toxin-activatable GTP-binding proteins, and causes the release of some of these α subunits from the brush border membrane so that they can interact with the catalytic subunit of adenylate cyclase in the basal-lateral membrane.

However, it should be noted that no control experiments were carried out to determine the amount of release of $G_{s\alpha}$, if any, that occurs in the absence of cholera toxin. Although, as mentioned earlier it has been found that no ADP-ribosylation of $G_{s\alpha}$ occurs in the absence of the toxin (results not shown), and so any toxin-independent release would not show up on the autoradiograph. As far as the radioactive material which is present at the top of the gel shown in Figure 4-5 is concerned there does not seem to be any immediate explanation. This would usually be indicative of aggregated protein, but since the radioactive material is only present in the soluble fractions (Fig. 4-5B lanes 3 & 5) and not in the pelleted fractions (Fig. 4-5B lanes 2 & 4) nor in the total labelling track (Fig. 4-5B lane 1) this cannot be the case here. This view is also supported by the absence of any aggregated material at the top of the gel (Fig. 4-5A lanes 3 & 5).

4.4 Effect of antidiarrhoeal drugs on the ADP-ribosylation of membrane proteins

4.4.1 Method

5 μM-[adenylate-^{32}P]NAD$^+$ and 20 μg/ml preactivated cholera toxin were incubated with 50 μg membrane protein and various concentrations (0.1, 0.2, 0.4, 0.7 and 1.0 mM) of the drugs shown in Figure 4-1, in a reaction volume of 0.1 ml at 30°C for 30 min, as in section 2.2.2.1. All of the drugs were freshly prepared for each experiment, as a 5 mM stock solution, by solubilization in double-distilled water because they were very light sensitive, and they oxidized on prolonged exposure to air, acquiring a blue or pink colour.

4.4.2 Results and discussion

From the start of this project it had been decided to look at the effect of the antidiarrhoeal drugs on the activation of adenylate cyclase and the phosphorylation of proteins in the membrane fractions since there seemed to be a good possibility that they would have an effect on these processes, as explained earlier in Chapter One (sections 1.4.3 and 1.4.4). Briefly, the movement of ions (Na^+-Cl^- absorption and Cl^- secretion) across the intestinal epithelium (Kimberg, 1974; Field, 1976) may be mediated by cyclic AMP (as well as cyclic GMP and Ca^{2+}), resulting from an increase in the activity of adenylate cyclase, and exerting its effect through the activation of cyclic-AMP-dependent protein kinase (Alhanaty and Shaltiel, 1979). The phenothiazine antidiarrhoeal drugs have been found to bind to calmodulin in a Ca^{2+}-dependent manner, whereas Ca^{2+}-

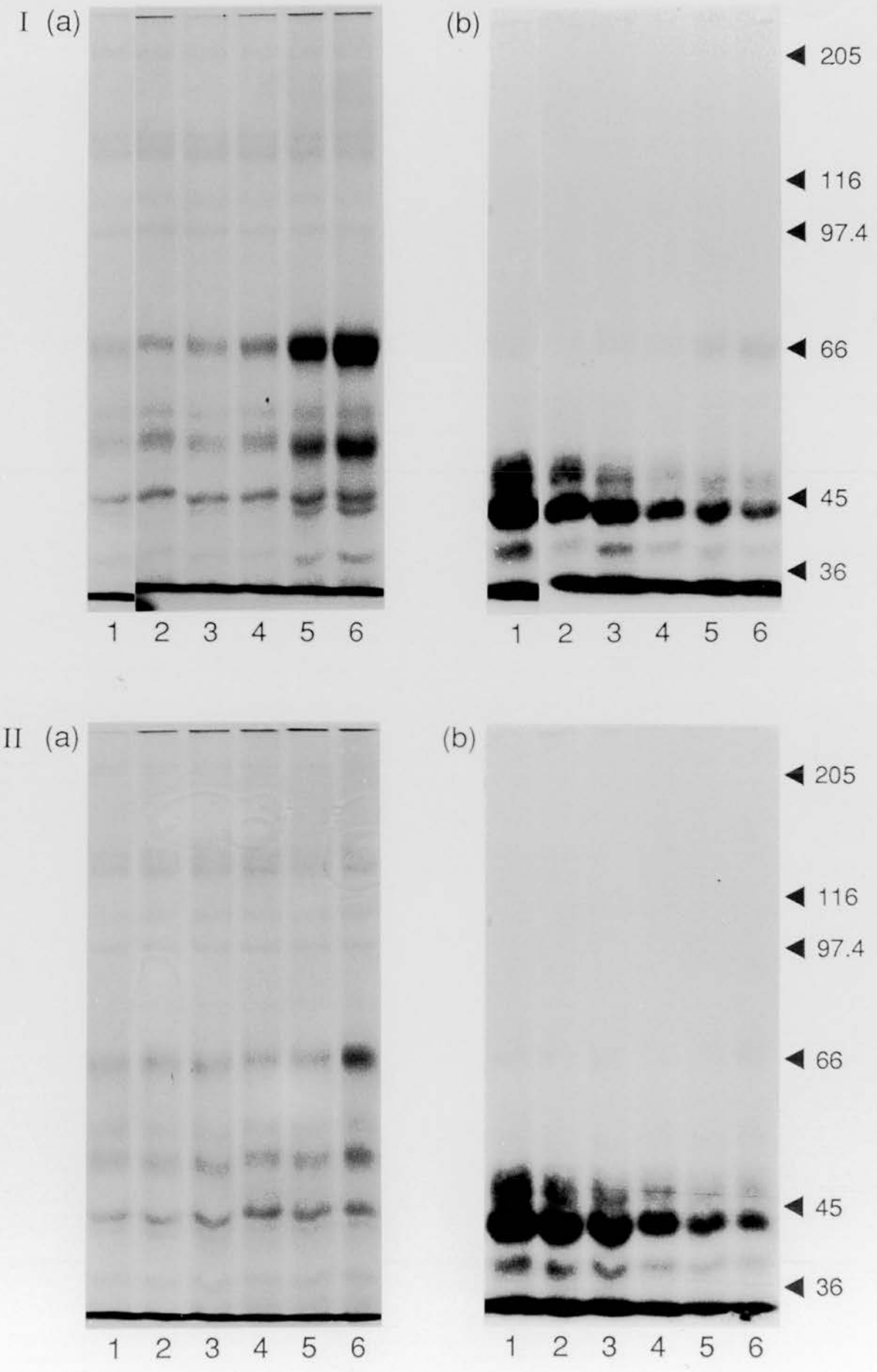
activated calmodulin has been found to activate a number of Ca^{2+}-dependent enzymes, including adenylate cyclase (Amiranoff *et al.*, 1983) and protein kinases (Kennedy and Greengard, 1981). Therefore, it has been proposed that the drugs bind calmodulin and reduce the activity of both adenylate cyclase and protein kinase (Levin and Weiss, 1977, 1978; Weiss and Levin, 1978). However, there was no reason to believe that the drugs would have any effect on the ADP-ribosylation of membrane proteins, but since the effects on adenylate cyclase activity and the phosphorylation of membrane proteins were going to be determined it seemed that any effects on ADP-ribosylation might as well be also investigated.

The resulting gel profiles and autoradiographs from the experiments are shown in Figure 4-6 and the graphs resulting from the densitometric analysis of the autoradiographs are shown in Figure 4-7. The effects of the six drugs on each membrane fraction were investigated separately. This means that although the labelling in each fraction for a particular drug cannot be compared, the labelling for each drug in a particular fraction can be. However, this does not really matter since the labelling between fractions has been previously compared (see Fig. 4-3 and Table 4-1). Figures 4-6 and 4-7 show quite clearly that the drugs do inhibit the cholera toxin induced ADP-ribosylation of the 45, 40 and 37 kDa membrane proteins.

Fig. 4-6 Effect of antidiarrhoeal drugs on the ADP-ribosylation of membrane proteins

(a) and (b) show the protein profiles (on 10% polyacrylamide gels) and corresponding autoradiographs respectively which are produced when **mixed plasma membranes (A)**, brush border membranes (B), and basal-lateral membranes (C) are incubated with 20 μg/ml preactivated cholera toxin, 5 μM-[adenylate-^{32}P]NAD^{+} and 0-1 mM drug for 30 min at 30°C as in section 4.4.1. Each lane contained 50 μg membrane protein. The drugs tested were **chlorpromazine (I), amitriptyline (II)**, trifluoperazine (III), promethazine (IV), triflupromazine (V) and promazine (VI). The migration of molecular mass standards expressed in kDa is as indicated. The concentration of drug used was as follows; no drug (lane 1), 0.1 mM (lane 2), 0.2 mM (lane 3), 0.4 mM (lane 4), 0.7 mM (lane 5) and 1.0 mM (lane 6).

A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(b)
205
116
97.4
66
45
36
1 2 3 4 5 6
1 2 3 4 5 6
II (a)
(b)
205
116
97.4
66
45
36
1 2 3 4 5 6
1 2 3 4 5 6

A

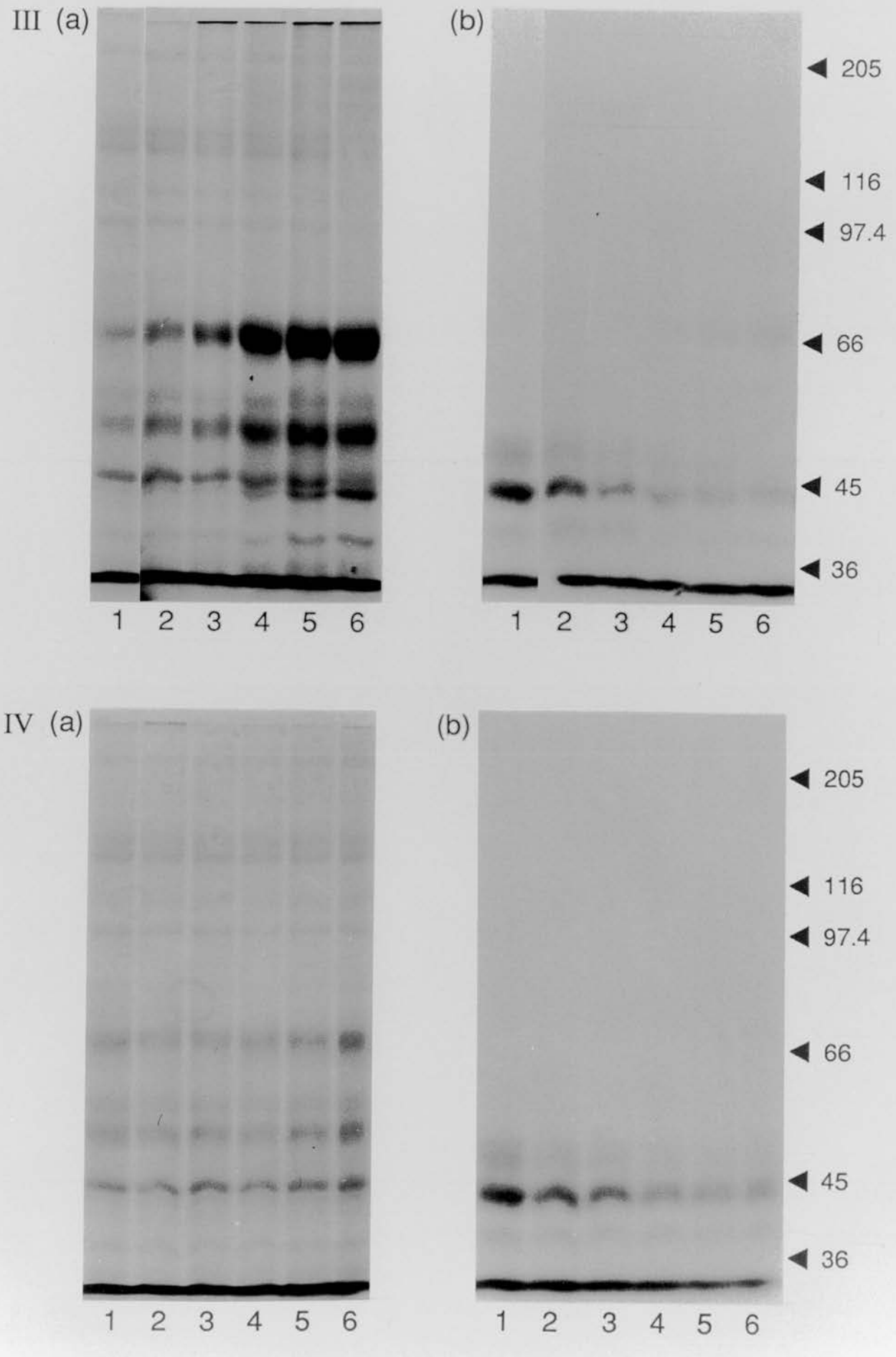
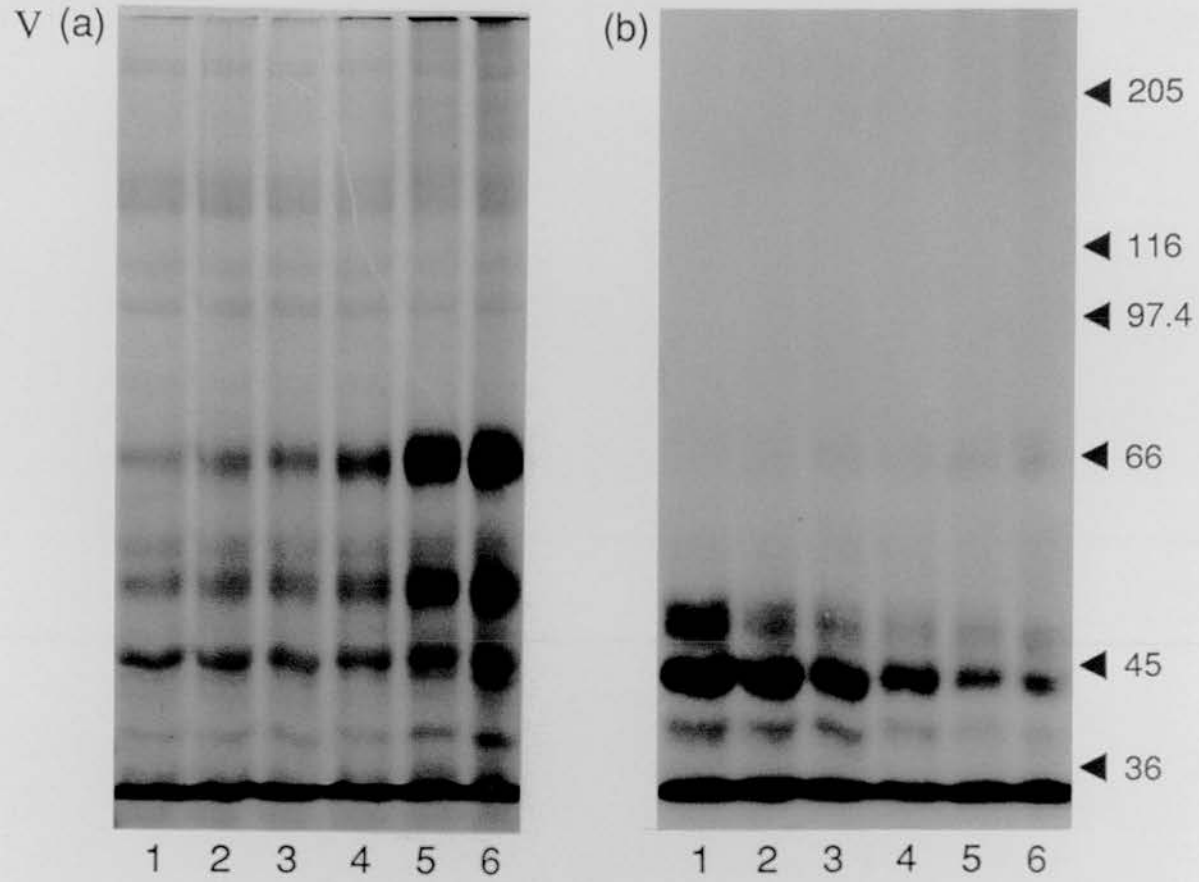

III (a)
(b)
205
116
97.4
66
45
36
1 2 3 4 5 6
1 2 3 4 5 6
IV (a)
(b)
205
116
97.4
66
45
36
1 2 3 4 5 6
1 2 3 4 5 6

Fig. 4-6 Effect of antidiarrhoeal drugs on the ADP-ribosylation of membrane proteins

(a) and (b) show the protein profiles (on 10% polyacrylamide gels) and corresponding autoradiographs respectively which are produced when **mixed plasma membranes (A)**, brush border membranes (B), and basal-lateral membranes (C) are incubated with 20 μg/ml preactivated cholera toxin, 5 μM-[adenylate-^{32}P]NAD^{+} and 0-1 mM drug for 30 min at 30°C as in section 4.4.1. Each lane contained 50 μg membrane protein. The drugs tested were chlorpromazine (I), amitriptyline (II), trifluoperazine (III), promethazine (IV), **triflupromazine (V)** and **promazine (VI)**. The migration of molecular mass standards expressed in kDa is as indicated. The concentration of drug used was as follows; no drug (lane 1), 0.1 mM (lane 2), 0.2 mM (lane 3), 0.4 mM (lane 4), 0.7 mM (lane 5) and 1.0 mM (lane 6).

A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(b)
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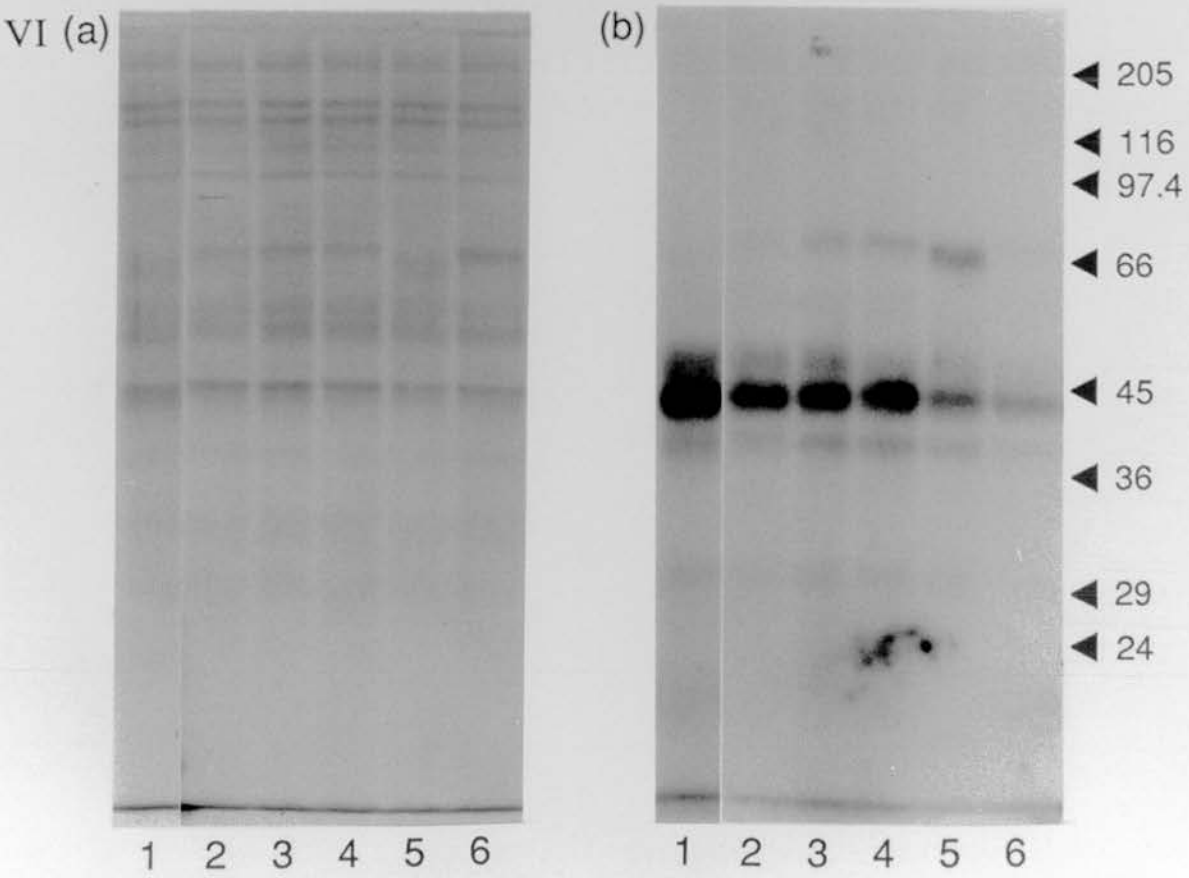
116
97.4
66
45
36
1 2 3 4 5 6
1 2 3 4 5 6


VI (a)
(b)
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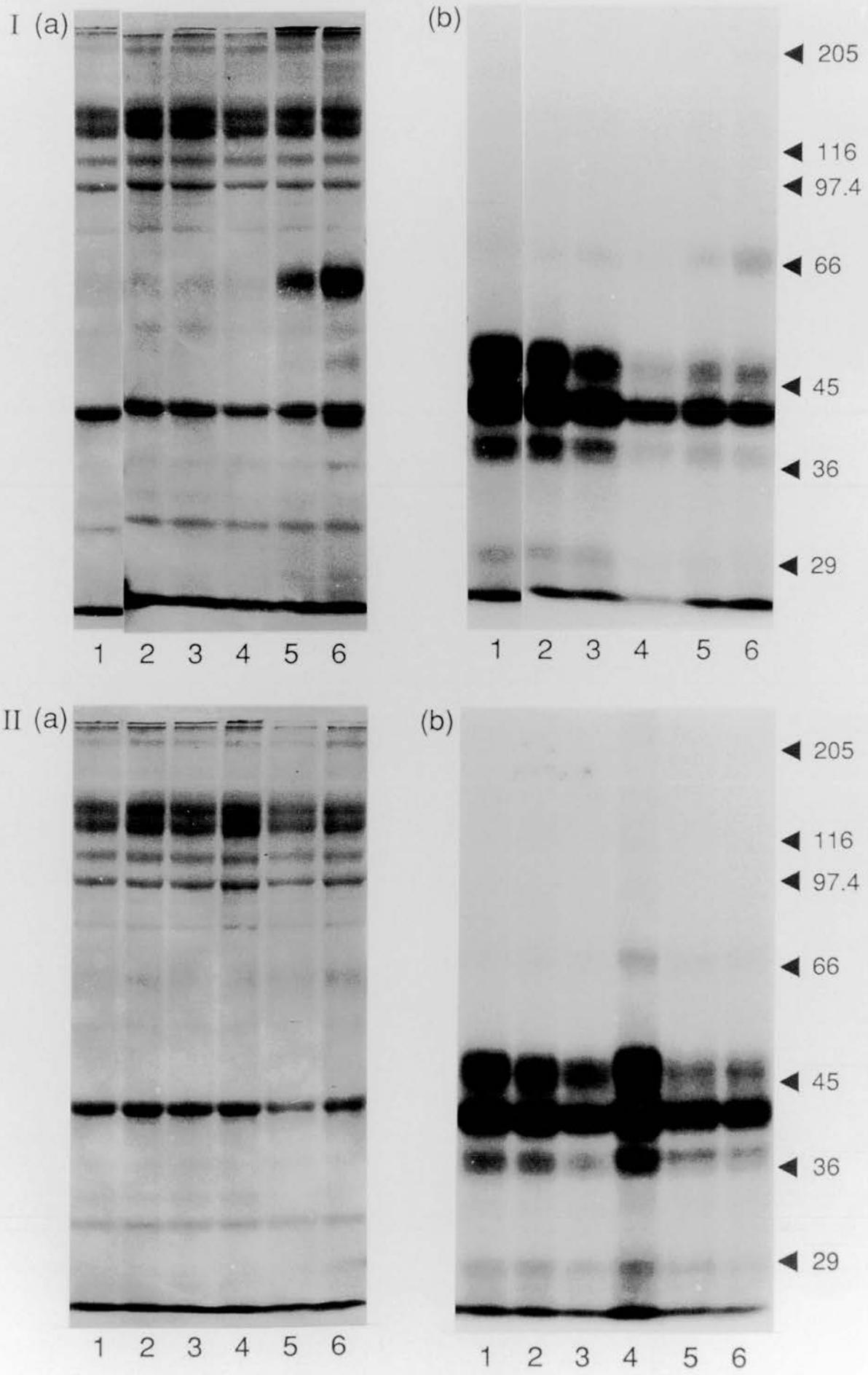
116
97.4
66
45
36
29
24
1 2 3 4 5 6
1 2 3 4 5 6

Fig. 4-6 Effect of antidiarrhoeal drugs on the ADP-ribosylation of membrane proteins

(a) and (b) show the protein profiles (on 10% polyacrylamide gels) and corresponding autoradiographs respectively which are produced when mixed plasma membranes (A), **brush border membranes (B)**, and basal-lateral membranes (C) are incubated with 20 μg/ml preactivated cholera toxin, 5 μM-[adenylate-^{32}P]NAD^{+} and 0-1 mM drug for 30 min at 30°C as in section 4.4.1. Each lane contained 50 μg membrane protein. The drugs tested were **chlorpromazine (I), amitriptyline (II)**, trifluoperazine (III), promethazine (IV), triflupromazine (V) and promazine (VI). The migration of molecular mass standards expressed in kDa is as indicated. The concentration of drug used was as follows; no drug (lane 1), 0.1 mM (lane 2), 0.2 mM (lane 3), 0.4 mM (lane 4), 0.7 mM (lane 5) and 1.0 mM (lane 6).

B


I (a)
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1 2 3 4 5 6
II (a)
(b)
205
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97.4
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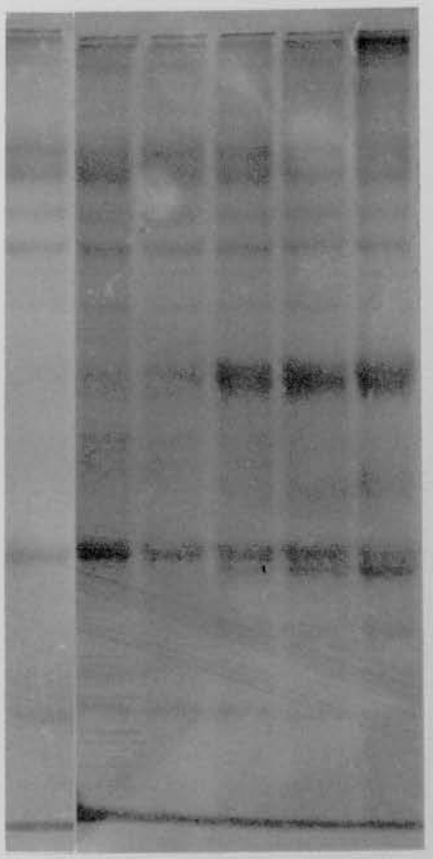
1 2 3 4 5 6

Fig. 4-6 Effect of antidiarrhoeal drugs on the ADP-ribosylation of membrane proteins

(a) and (b) show the protein profiles (on 10% polyacrylamide gels) and corresponding autoradiographs respectively which are produced when mixed plasma membranes (A), **brush border membranes (B)**, and basal-lateral membranes (C) are incubated with 20 μg/ml preactivated cholera toxin, 5 μM-[adenylate-^{32}P]NAD^{+} and 0-1 mM drug for 30 min at 30°C as in section 4.4.1. Each lane contained 50 μg membrane protein. The drugs tested were chlorpromazine (I), amitriptyline (II), **trifluoperazine (III), promethazine (IV),** triflupromazine (V) and promazine (VI). The migration of molecular mass standards expressed in kDa is as indicated. The concentration of drug used was as follows; no drug (lane 1), 0.1 mM (lane 2), 0.2 mM (lane 3), 0.4 mM (lane 4), 0.7 mM (lane 5) and 1.0 mM (lane 6).

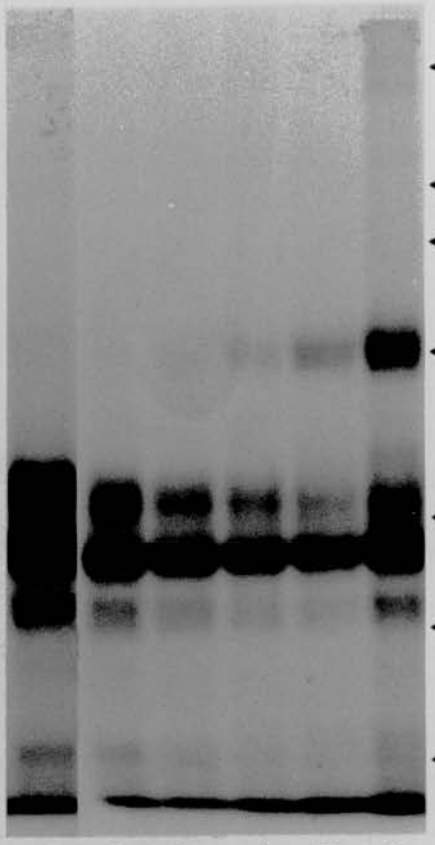
B

III (a)



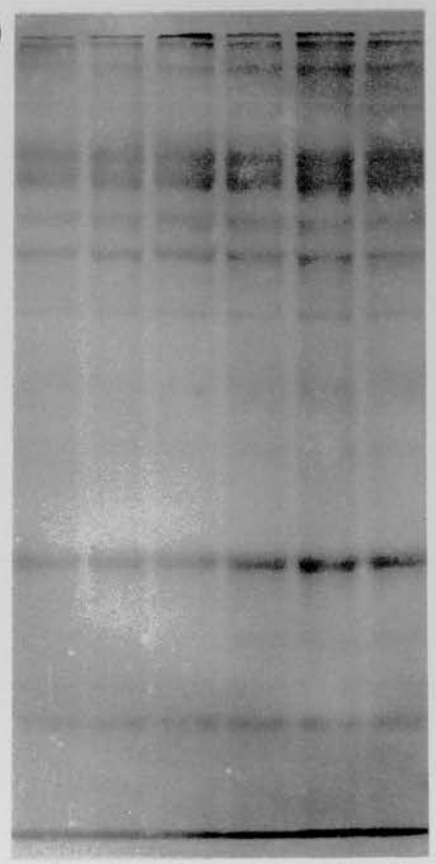
1 2 3 4 5 6

(b)



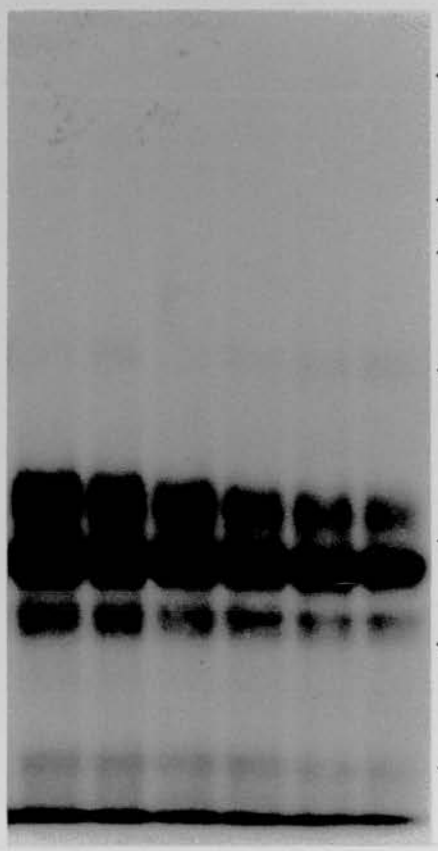
1 2 3 4 5 6

IV (a)



1 2 3 4 5 6

(b)

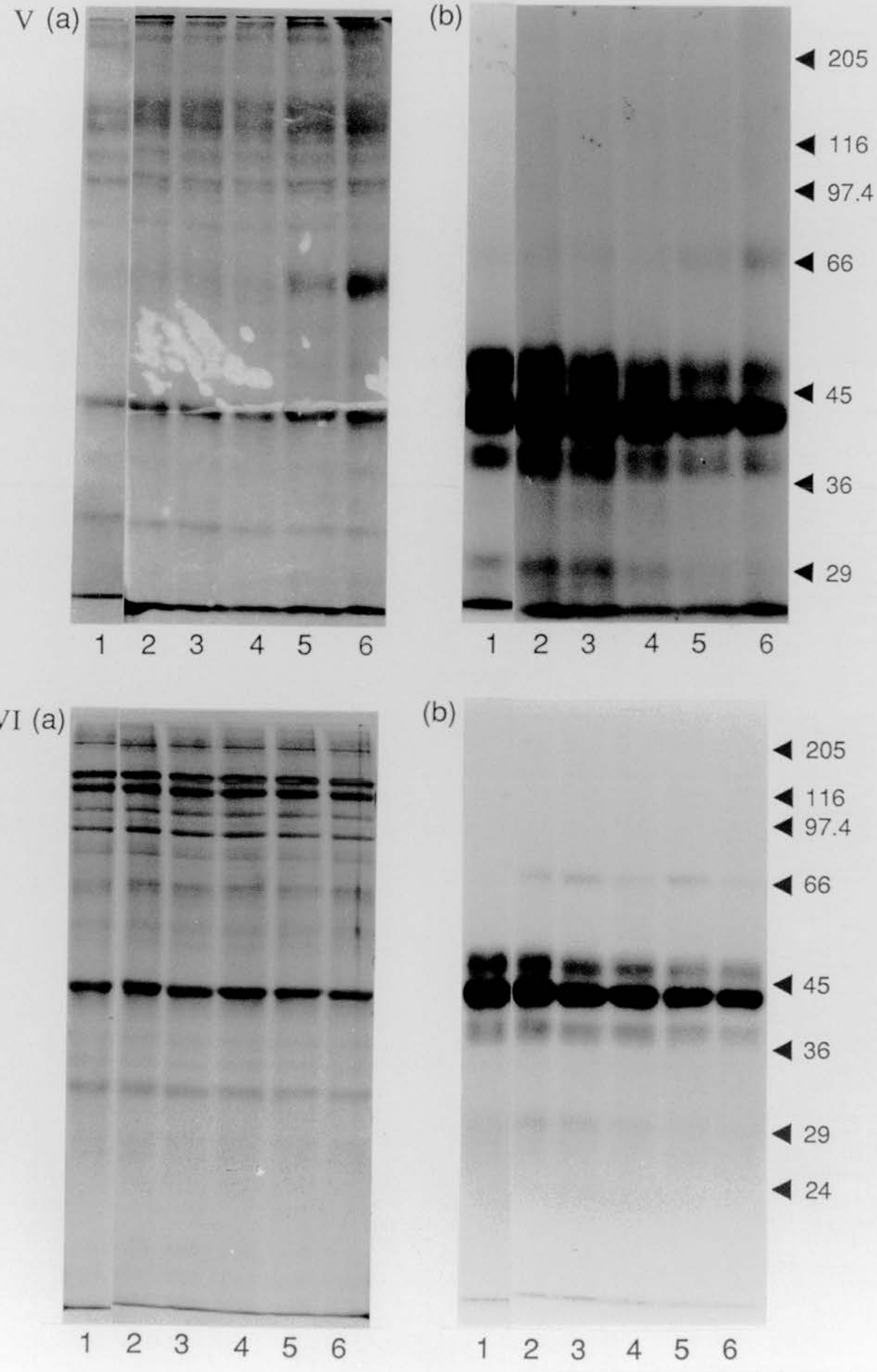


1 2 3 4 5 6

Fig. 4-6 Effect of antidiarrhoeal drugs on the ADP-ribosylation of membrane proteins

(a) and (b) show the protein profiles (on 10% polyacrylamide gels) and corresponding autoradiographs respectively which are produced when mixed plasma membranes (A), **brush border membranes (B)**, and basal-lateral membranes (C) are incubated with 20 μg/ml preactivated cholera toxin, 5 μM-[adenylate-^{32}P]NAD^{+} and 0-1 mM drug for 30 min at 30°C as in section 4.4.1. Each lane contained 50 μg membrane protein. The drugs tested were chlorpromazine (I), amitriptyline (II), trifluoperazine (III), promethazine (IV), **triflupromazine (V)** and **promazine (VI)**. The migration of molecular mass standards expressed in kDa is as indicated. The concentration of drug used was as follows; no drug (lane 1), 0.1 mM (lane 2), 0.2 mM (lane 3), 0.4 mM (lane 4), 0.7 mM (lane 5) and 1.0 mM (lane 6).

B


V (a)
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1 2 3 4 5 6
1 2 3 4 5 6
VI (a)
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1 2 3 4 5 6
1 2 3 4 5 6

Fig. 4-6 Effect of antidiarrhoeal drugs on the ADP-ribosylation of membrane proteins

(a) and (b) show the protein profiles (on 10% polyacrylamide gels) and corresponding autoradiographs respectively which are produced when mixed plasma membranes (A), brush border membranes (B), and **basal-lateral membranes (C)** are incubated with 20 μg/ml preactivated cholera toxin, 5 μM-[adenylate-^{32}P]NAD^{+} and 0-1 mM drug for 30 min at 30°C as in section 4.4.1. Each lane contained 50 μg membrane protein. The drugs tested were **chlorpromazine (I), amitriptyline (II)**, trifluoperazine (III), promethazine (IV), triflupromazine (V) and promazine (VI). The migration of molecular mass standards expressed in kDa is as indicated. The concentration of drug used was as follows; no drug (lane 1), 0.1 mM (lane 2), 0.2 mM (lane 3), 0.4 mM (lane 4), 0.7 mM (lane 5) and 1.0 mM (lane 6).

C

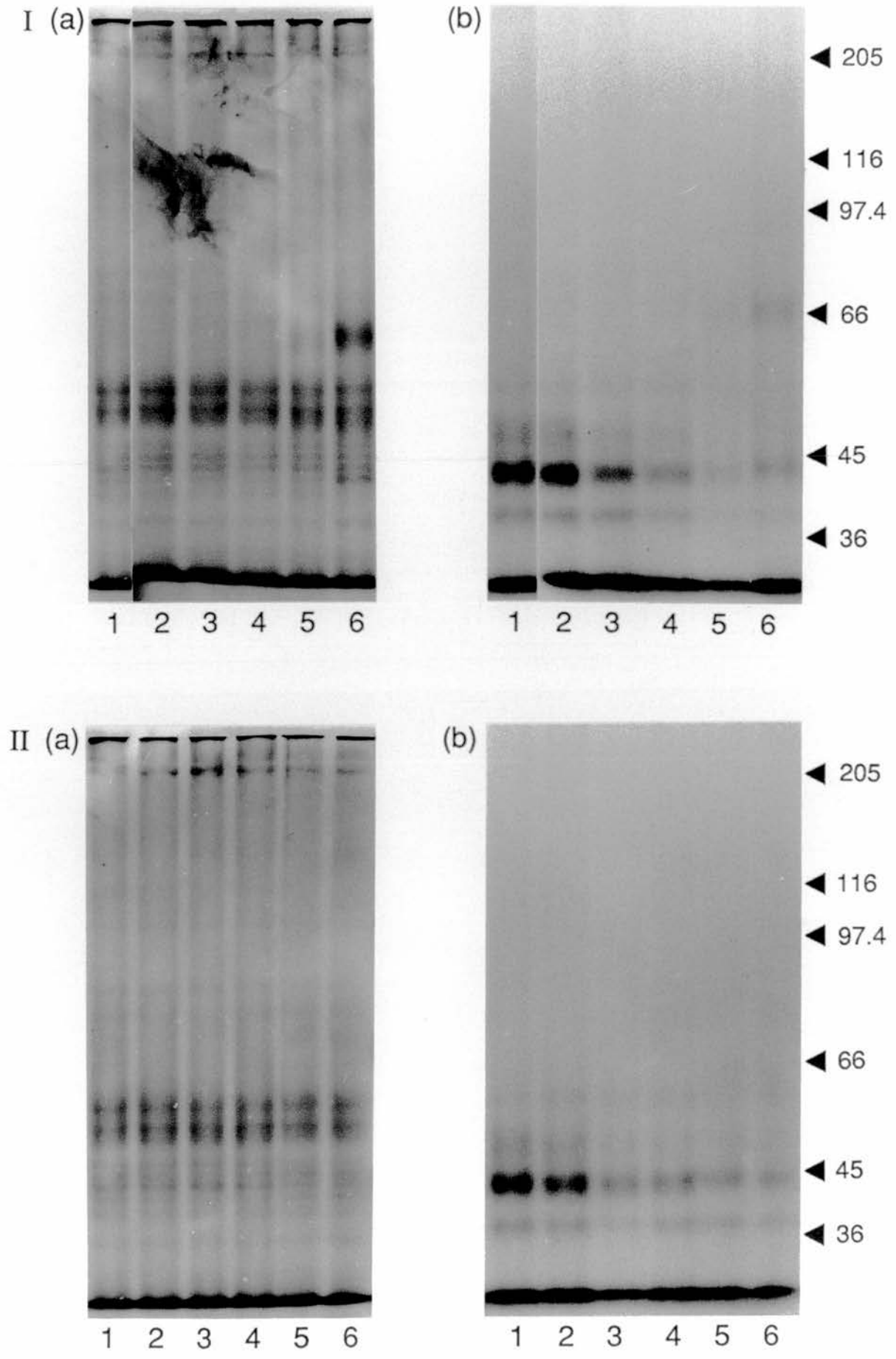


Fig. 4-6 Effect of antidiarrhoeal drugs on the ADP-ribosylation of membrane proteins

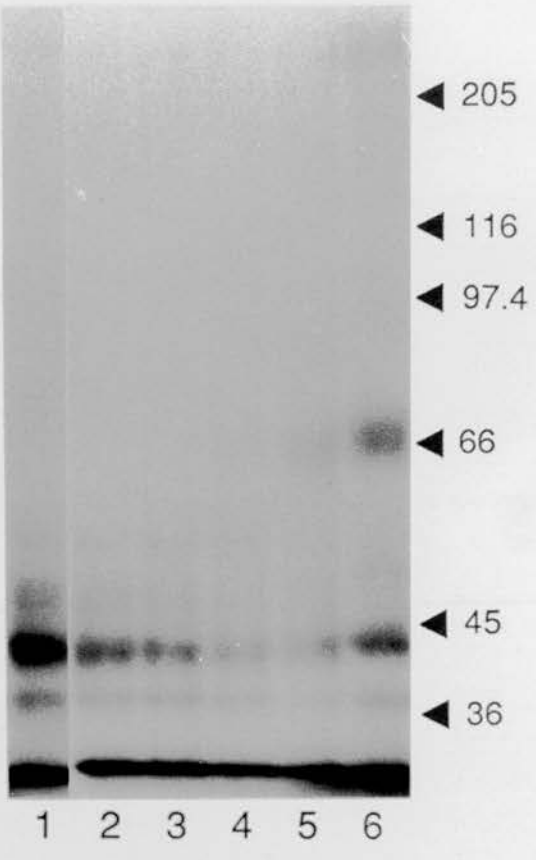
(a) and (b) show the protein profiles (on 10% polyacrylamide gels) and corresponding autoradiographs respectively which are produced when mixed plasma membranes (A), brush border membranes (B), and **basal-lateral membranes (C)** are incubated with 20 μg/ml preactivated cholera toxin, 5 μM-[adenylate-^{32}P]NAD^{+} and 0-1 mM drug for 30 min at 30°C as in section 4.4.1. Each lane contained 50 μg membrane protein. The drugs tested were chlorpromazine (I), amitriptyline (II), **trifluoperazine (III), promethazine (IV),** triflupromazine (V) and promazine (VI). The migration of molecular mass standards expressed in kDa is as indicated. The concentration of drug used was as follows; no drug (lane 1), 0.1 mM (lane 2), 0.2 mM (lane 3), 0.4 mM (lane 4), 0.7 mM (lane 5) and 1.0 mM (lane 6).

C

III (a)


1 2 3 4 5 6

(b)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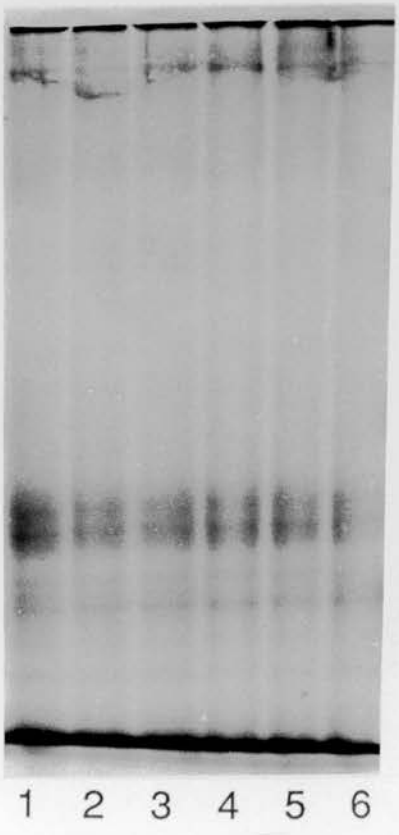
IV (a)


1 2 3 4 5 6

(b)

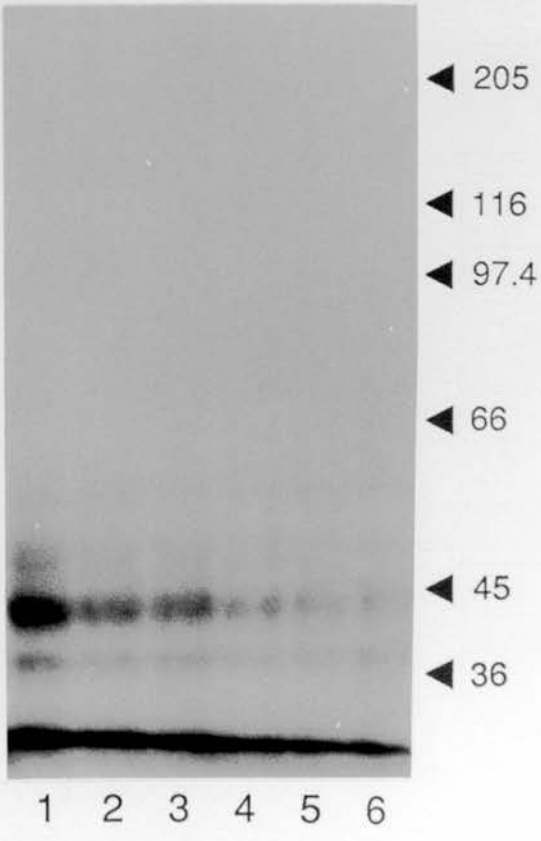

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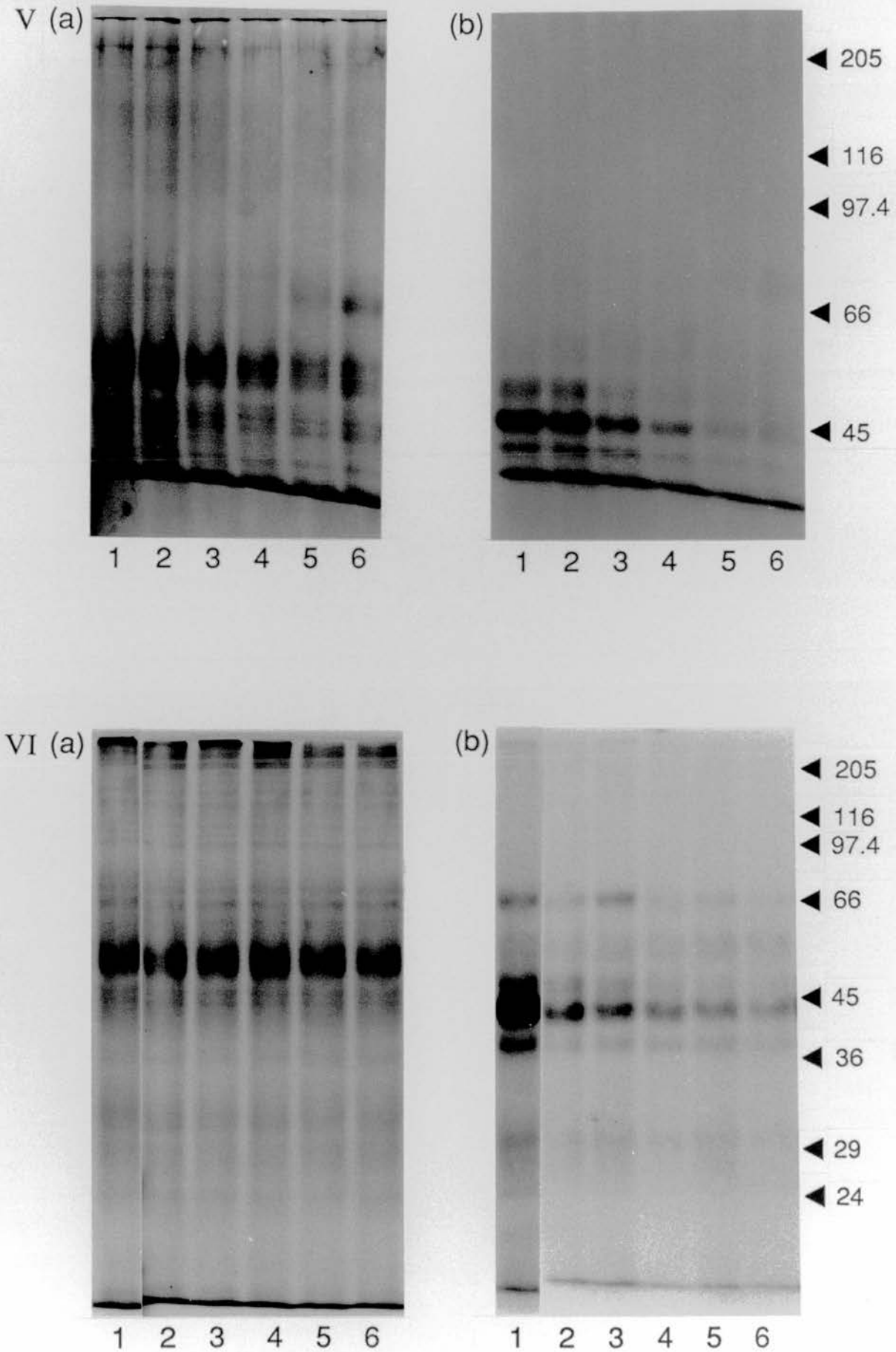
Fig. 4-6 Effect of antidiarrhoeal drugs on the ADP-ribosylation of membrane proteins

(a) and (b) show the protein profiles (on 10% polyacrylamide gels) and corresponding autoradiographs respectively which are produced when mixed plasma membranes (A), brush border membranes (B), and **basal-lateral membranes (C)** are incubated with 20 μg/ml preactivated cholera toxin, 5 μM-[adenylate-^{32}P]NAD^{+} and 0-1 mM drug for 30 min at 30°C as in section 4.4.1. Each lane contained 50 μg membrane protein. The drugs tested were chlorpromazine (I), amitriptyline (II), trifluoperazine (III), promethazine (IV), **triflupromazine (V)** and **promazine (VI)**. The migration of molecular mass standards expressed in kDa is as indicated. The concentration of drug used was as follows; no drug (lane 1), 0.1 mM (lane 2), 0.2 mM (lane 3), 0.4 mM (lane 4), 0.7 mM (lane 5) and 1.0 mM (lane 6).

C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1 2 3 4 5 6
1 2 3 4 5 6

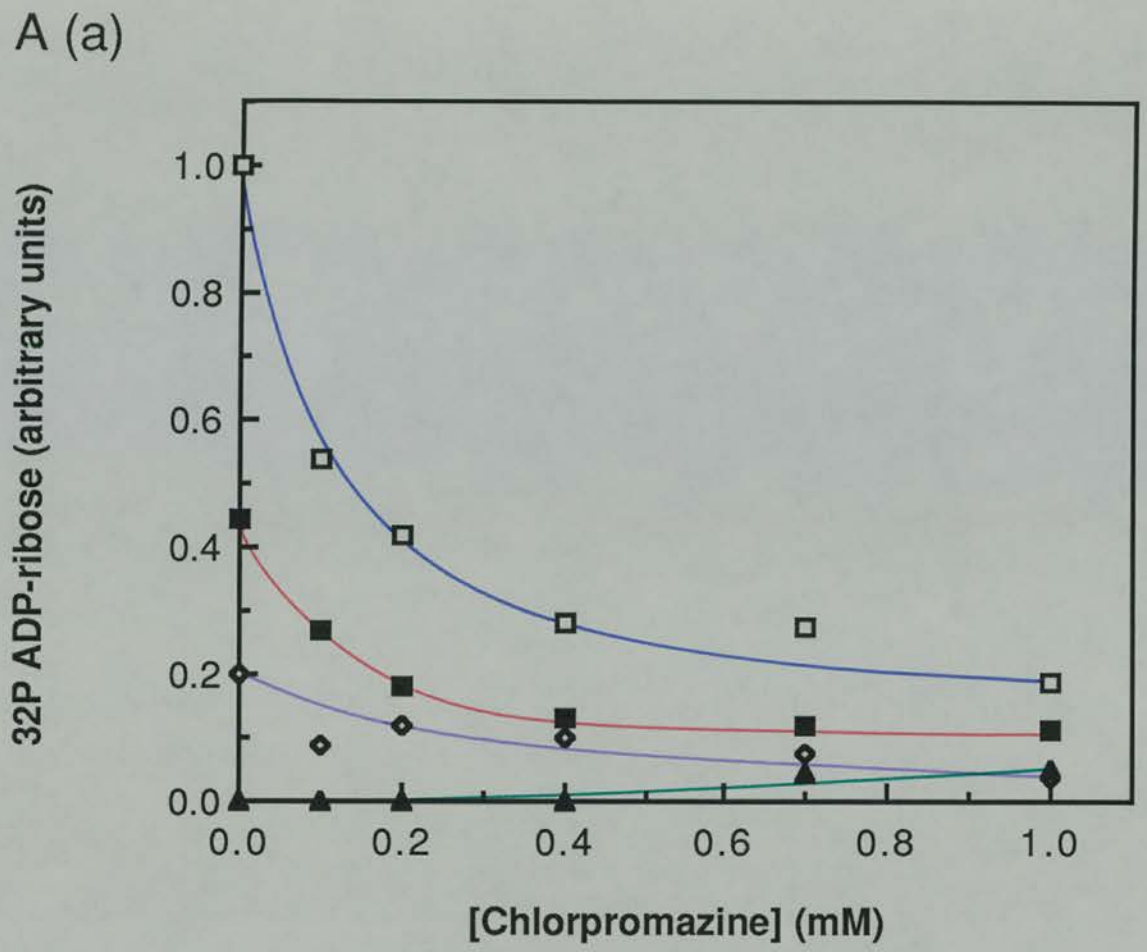
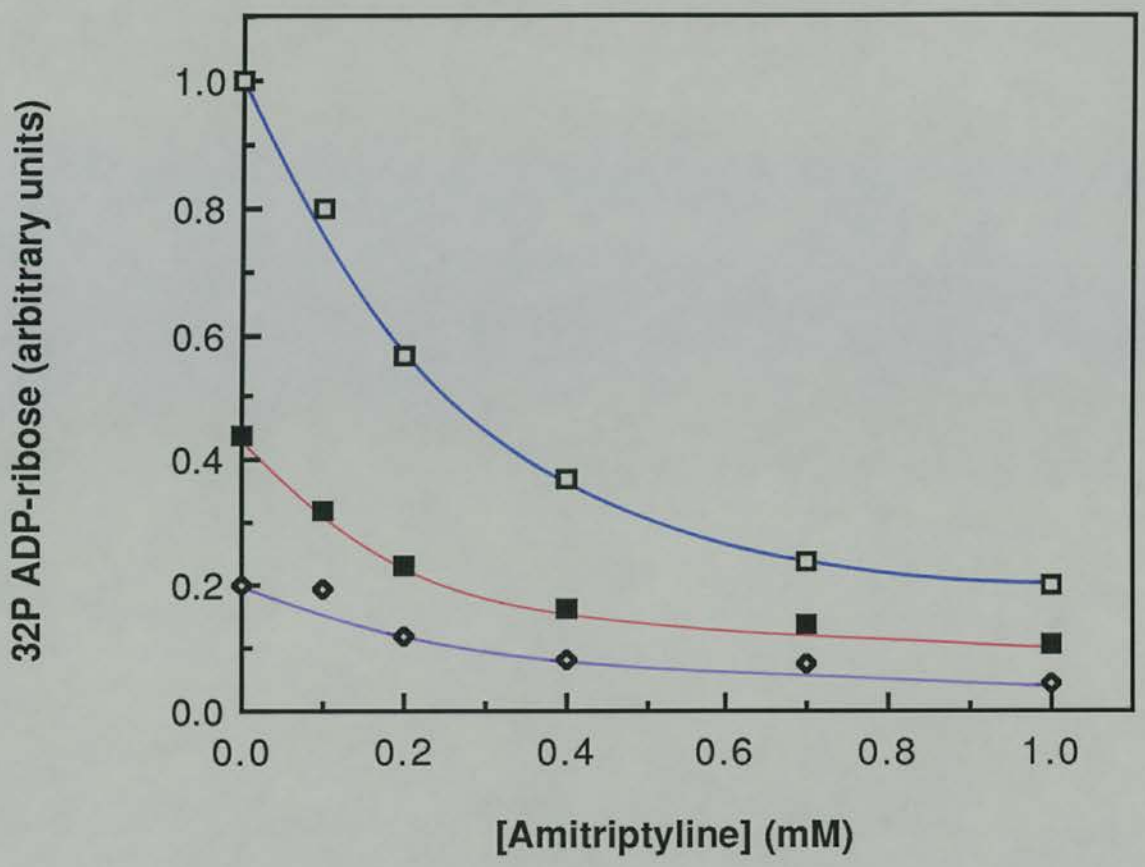


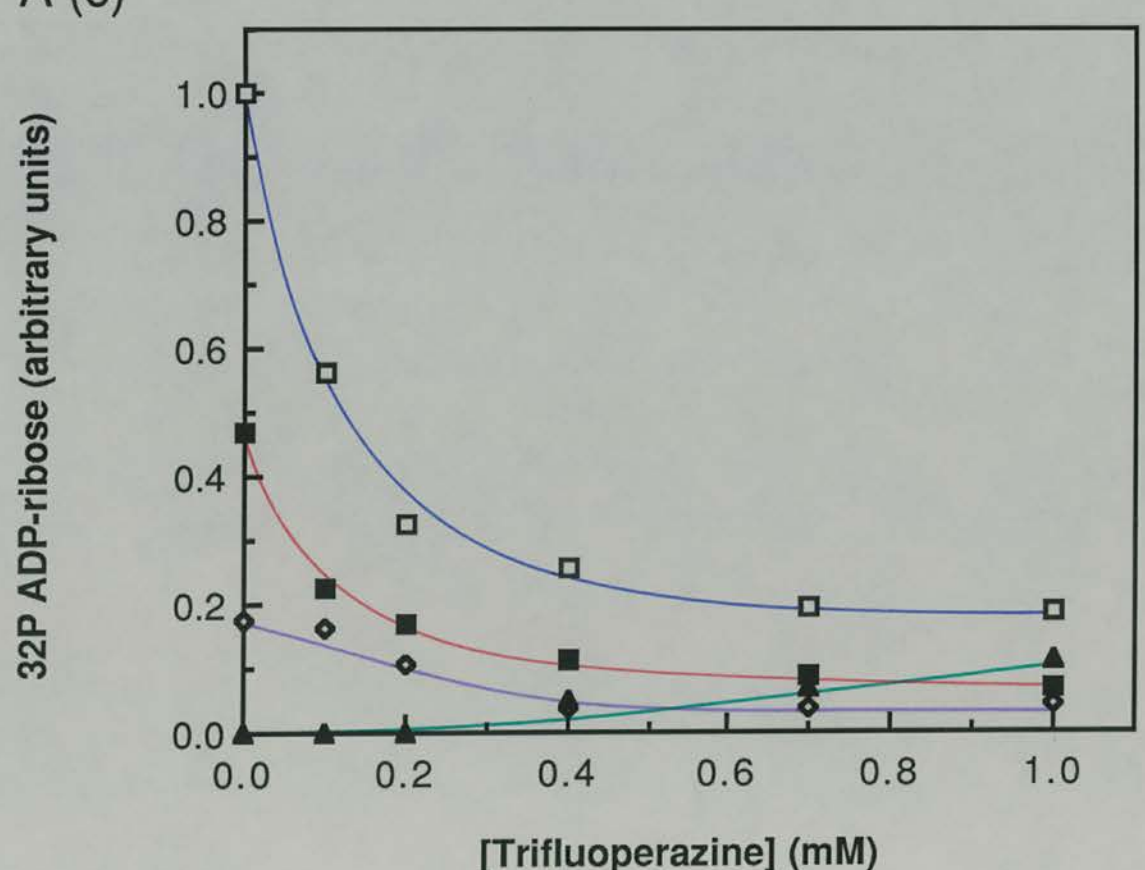
Fig. 4-7 **Effect of antidiarrhoeal drugs on the incorporation of ^{32}P-ADP-ribose into membrane proteins**

Graphs show the incorporation of ^{32}P-ADP-ribose into 40 kDa (□—□), 45 kDa (■—■), 37 kDa (◇—◇) and 66 kDa (▲—▲)proteins when **mixed plasma membranes (A)**, brush border membranes (B) and basal-lateral membranes (C) were incubated with 20 μg/ml preactivated cholera toxin, 5 μM-[adenylate-^{32}P]NAD$^+$ and 0-1 mM drug for 30 min at 30°C as in section 4.4.1. The drugs used were **chlorpromazine (a), amitriptyline (b), trifluoperazine (c)**, promethazine (d), triflupromazine (e) and promazine (f). The arbitrary unit of 1 for mixed plasma membranes, brush border membranes and basal-lateral membranes corresponds to integrated values of 30000, 60000 and 15000 respectively, obtained from the densitometric analysis of the autoradiographs (Fig. 4-6).

A (b)


32P ADP-ribose (arbitrary units)
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.2
0.4
0.6
0.8
1.0
[Amitriptyline] (mM)

A (c)


32P ADP-ribose (arbitrary units)
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.2
0.4
0.6
0.8
1.0
[Trifluoperazine] (mM)

A (d)

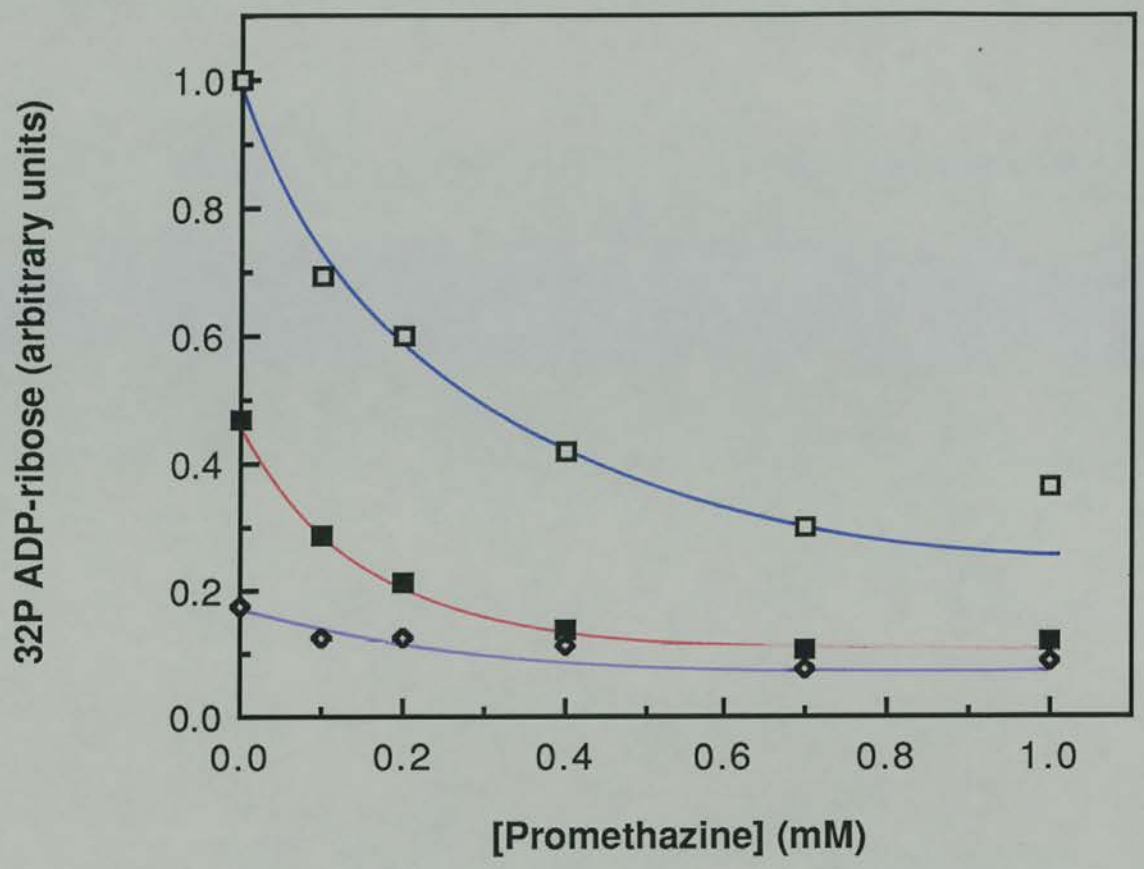
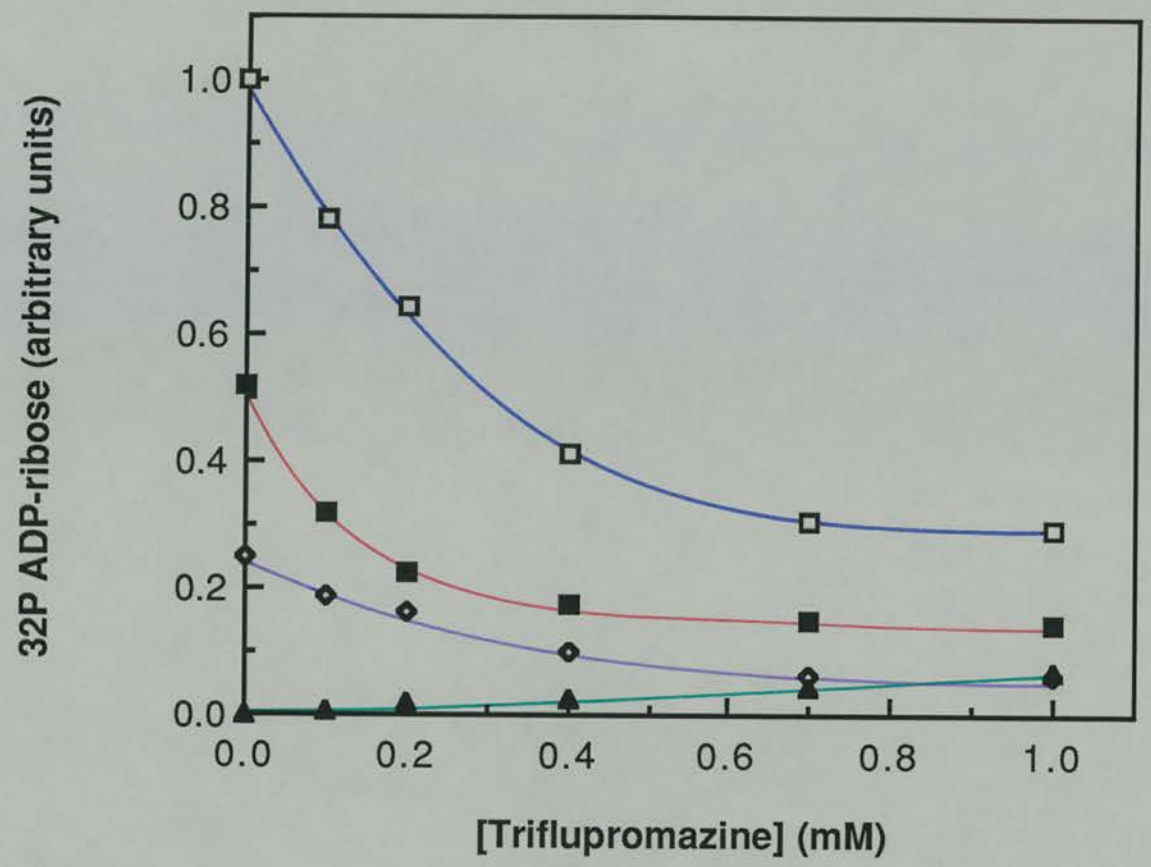


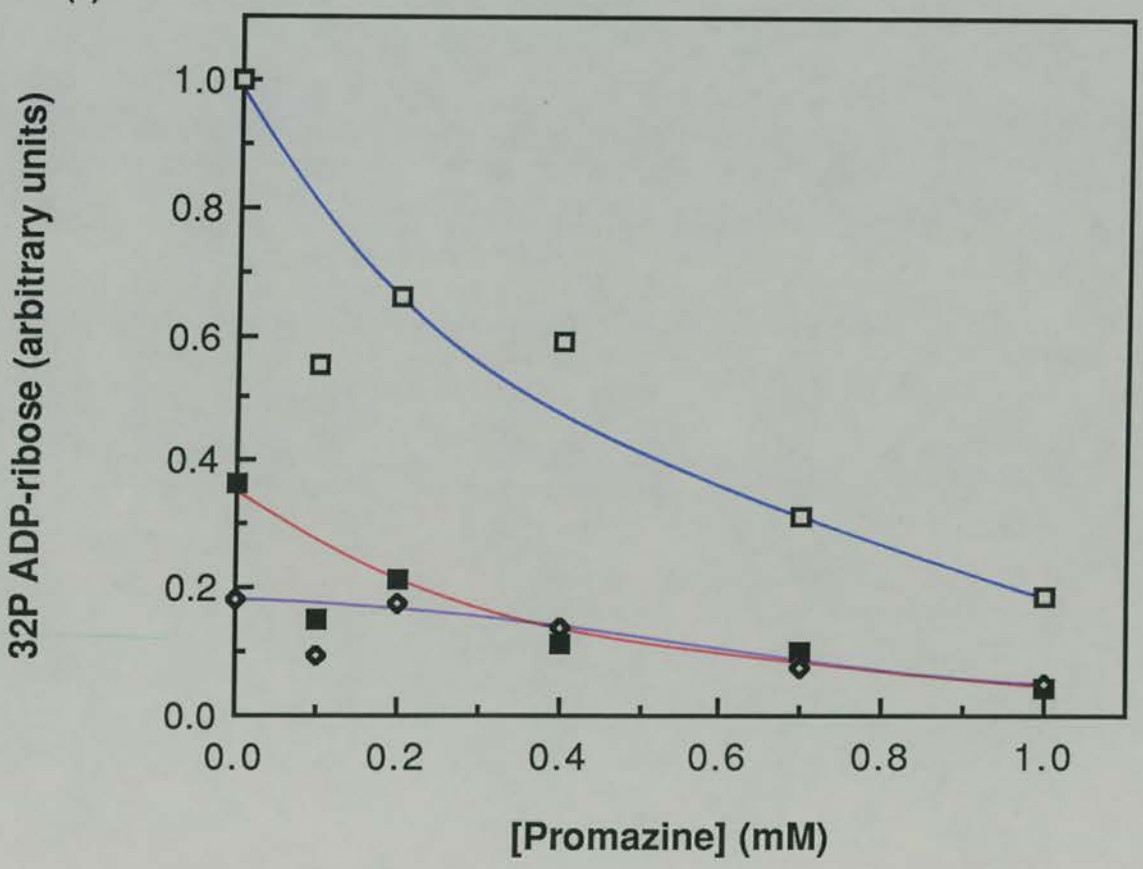
Fig. 4-7 **Effect of antidiarrhoeal drugs on the incorporation of ^{32}P-ADP-ribose into membrane proteins**

Graphs show the incorporation of ^{32}P-ADP-ribose into 40 kDa (□—□), 45 kDa (■—■), 37 kDa (◇—◇) and 66 kDa (▲—▲)proteins when **mixed plasma membranes (A)**, brush border membranes (B) and basal-lateral membranes (C) were incubated with 20 μg/ml preactivated cholera toxin, 5 μM-[adenylate-^{32}P]NAD$^+$ and 0-1 mM drug for 30 min at 30°C as in section 4.4.1. The drugs used were chlorpromazine (a), amitriptyline (b), trifluoperazine (c), **promethazine (d), triflupromazine (e)** and **promazine (f)**. The arbitrary unit of 1 for mixed plasma membranes, brush border membranes and basal-lateral membranes corresponds to integrated values of 30000, 60000 and 15000 respectively, obtained from the densitometric analysis of the autoradiographs (Fig. 4-6).

A (e)


32P ADP-ribose (arbitrary units)
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.2
0.4
0.6
0.8
1.0
[Trifluprom azine] (mM)

A (f)


32P ADP-ribose (arbitrary units)
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.2
0.4
0.6
0.8
1.0
[Promazine] (mM)

B (a)

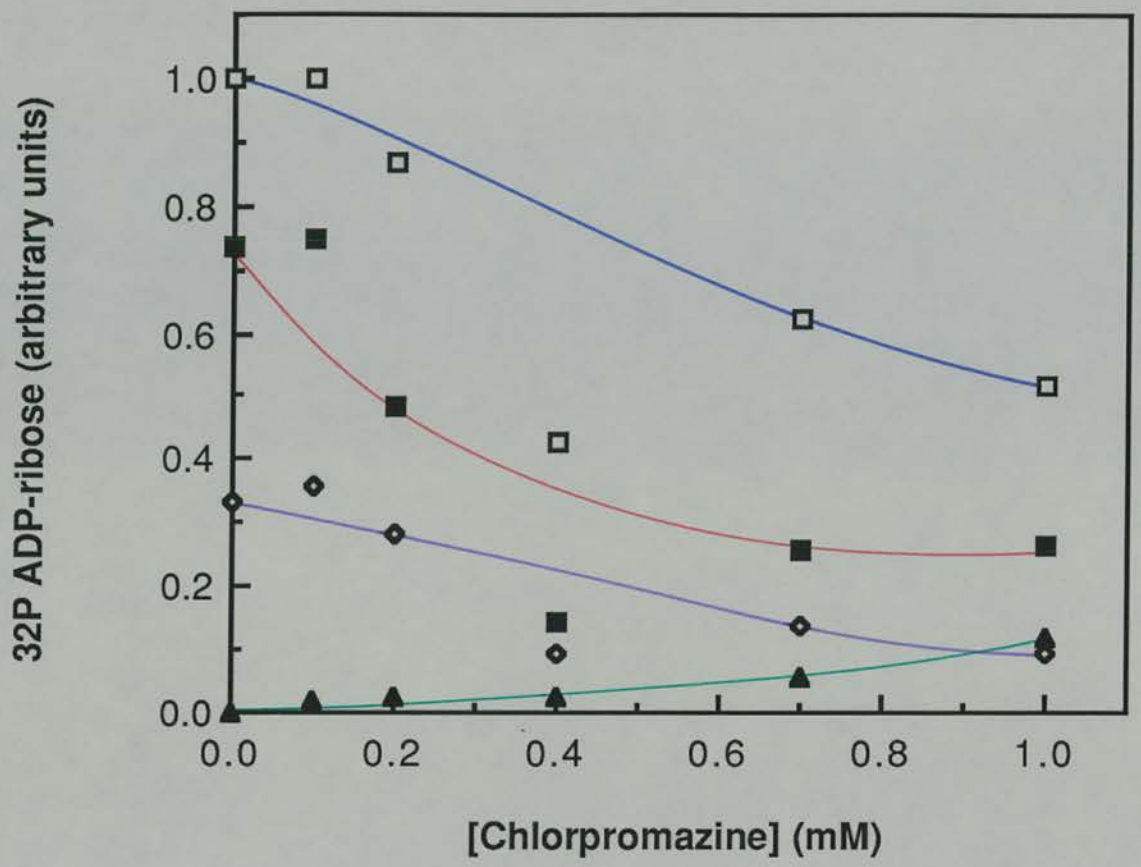
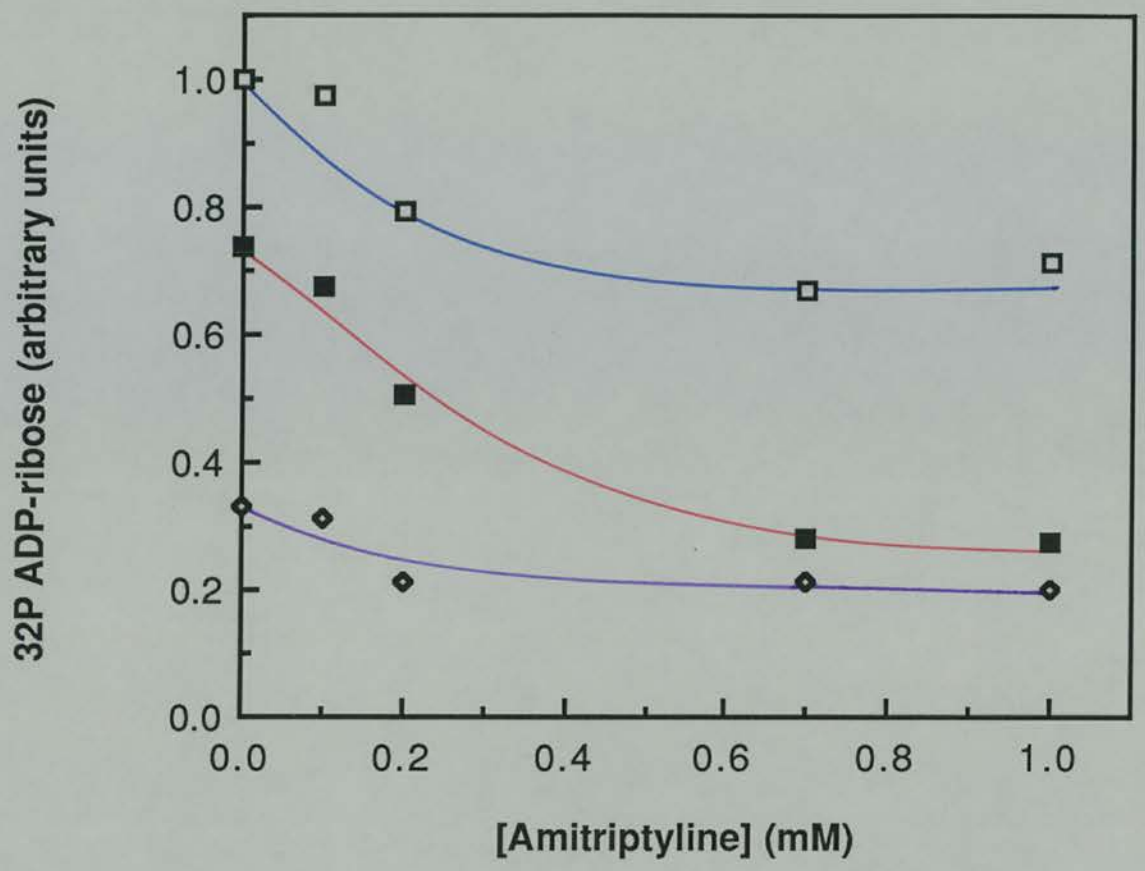


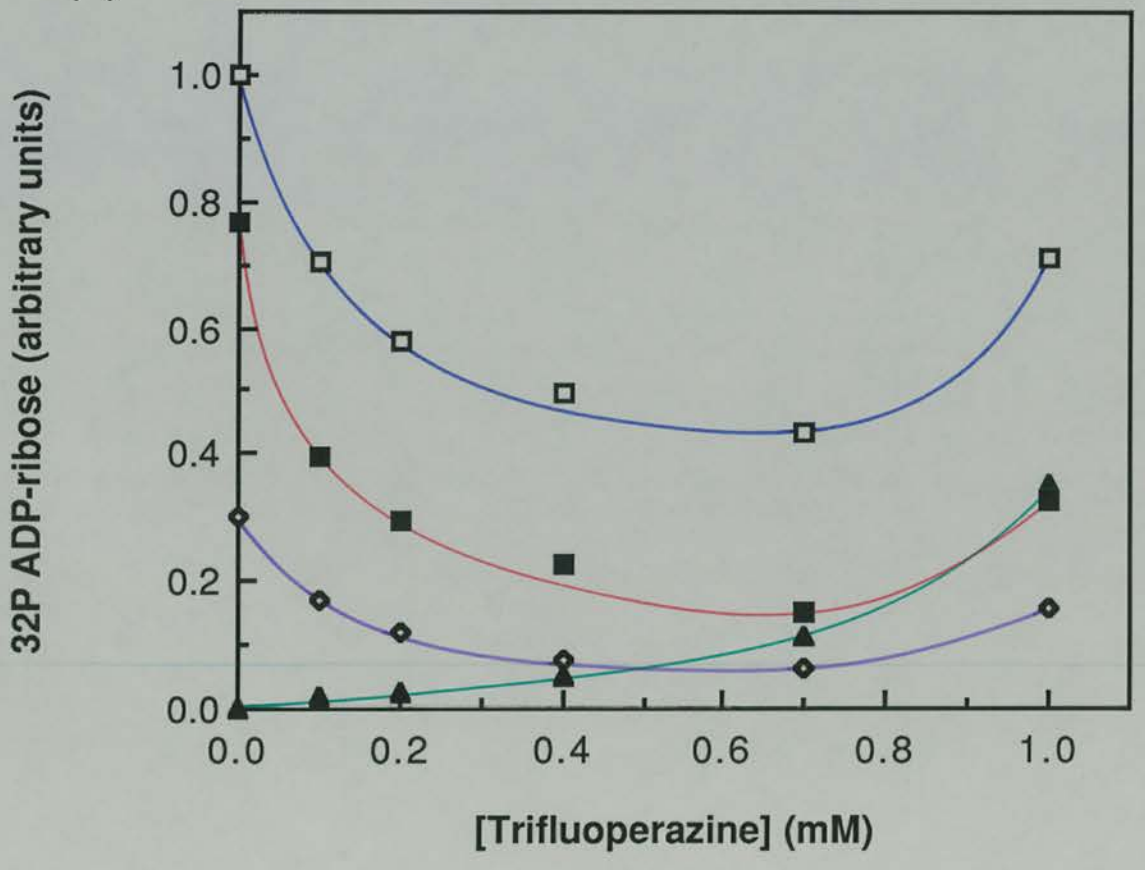
Fig. 4-7 **Effect of antidiarrhoeal drugs on the incorporation of ^{32}P-ADP-ribose into membrane proteins**

Graphs show the incorporation of ^{32}P-ADP-ribose into 40 kDa (□—□), 45 kDa (■—■), 37 kDa (◇—◇) and 66 kDa (▲—▲)proteins when mixed plasma membranes (A), **brush border membranes** (B) and basal-lateral membranes (C) were incubated with 20 μg/ml preactivated cholera toxin, 5 μM-[adenylate-^{32}P]NAD$^+$ and 0-1 mM drug for 30 min at 30°C as in section 4.4.1. The drugs used were **chlorpromazine (a), amitriptyline (b), trifluoperazine (c)**, promethazine (d), triflupromazine (e) and promazine (f). The arbitrary unit of 1 for mixed plasma membranes, brush border membranes and basal-lateral membranes corresponds to integrated values of 30000, 60000 and 15000 respectively, obtained from the densitometric analysis of the autoradiographs (Fig. 4-6).

B (b)


32P ADP-ribose (arbitrary units)
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.2
0.4
0.6
0.8
1.0
[Amitriptyline] (mM)

B (c)


32P ADP-ribose (arbitrary units)
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.2
0.4
0.6
0.8
1.0
[Trifluoperazine] (mM)

B (d)

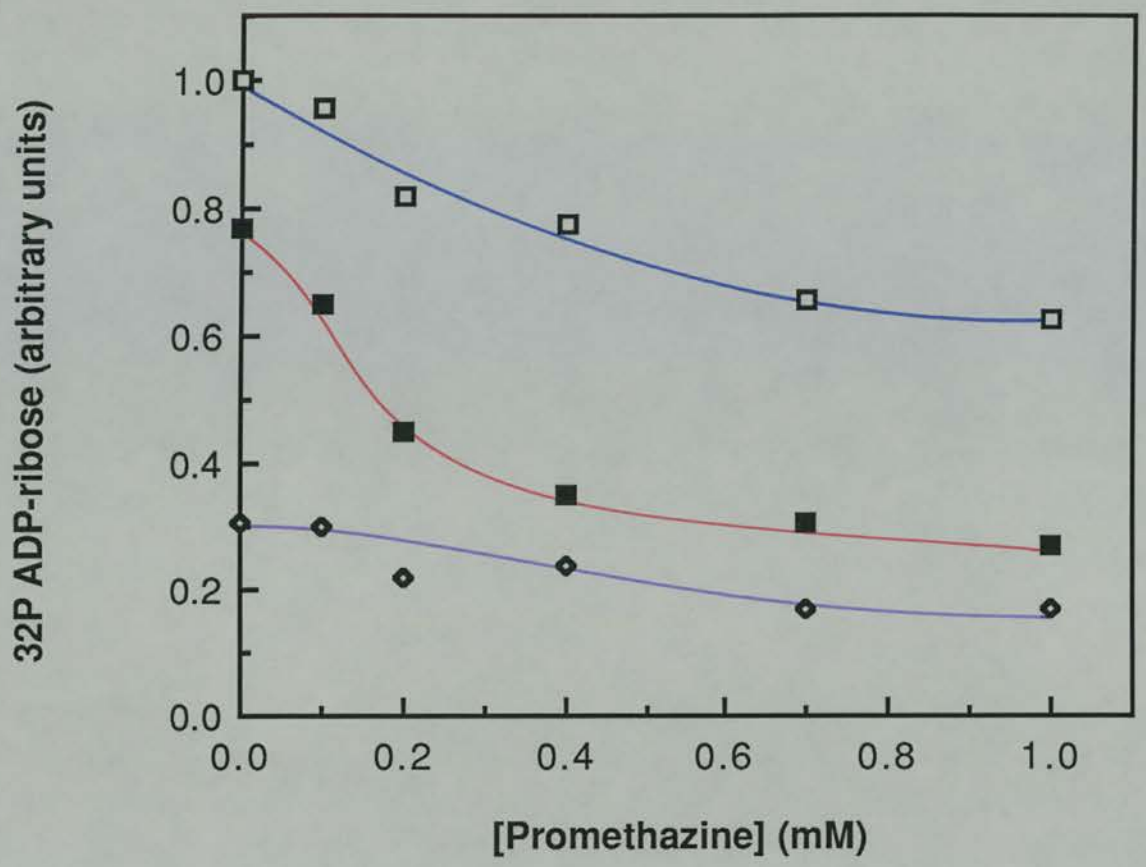
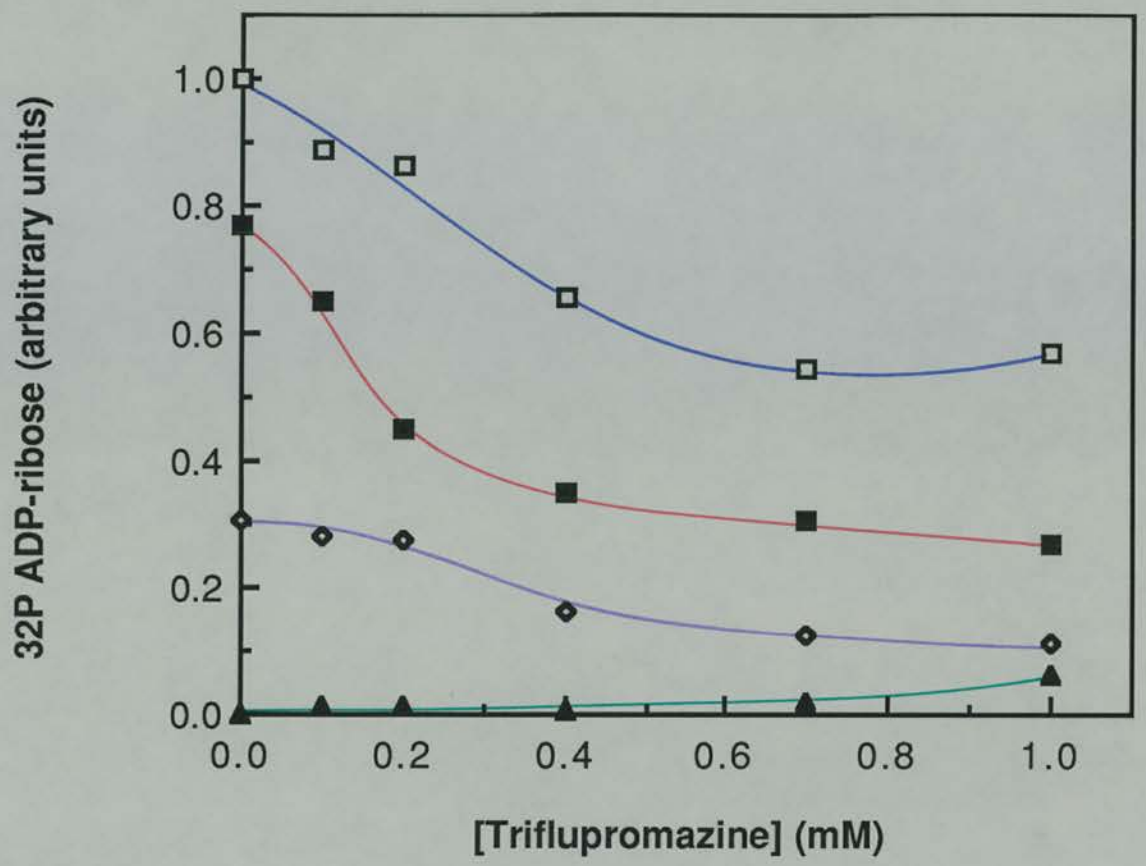


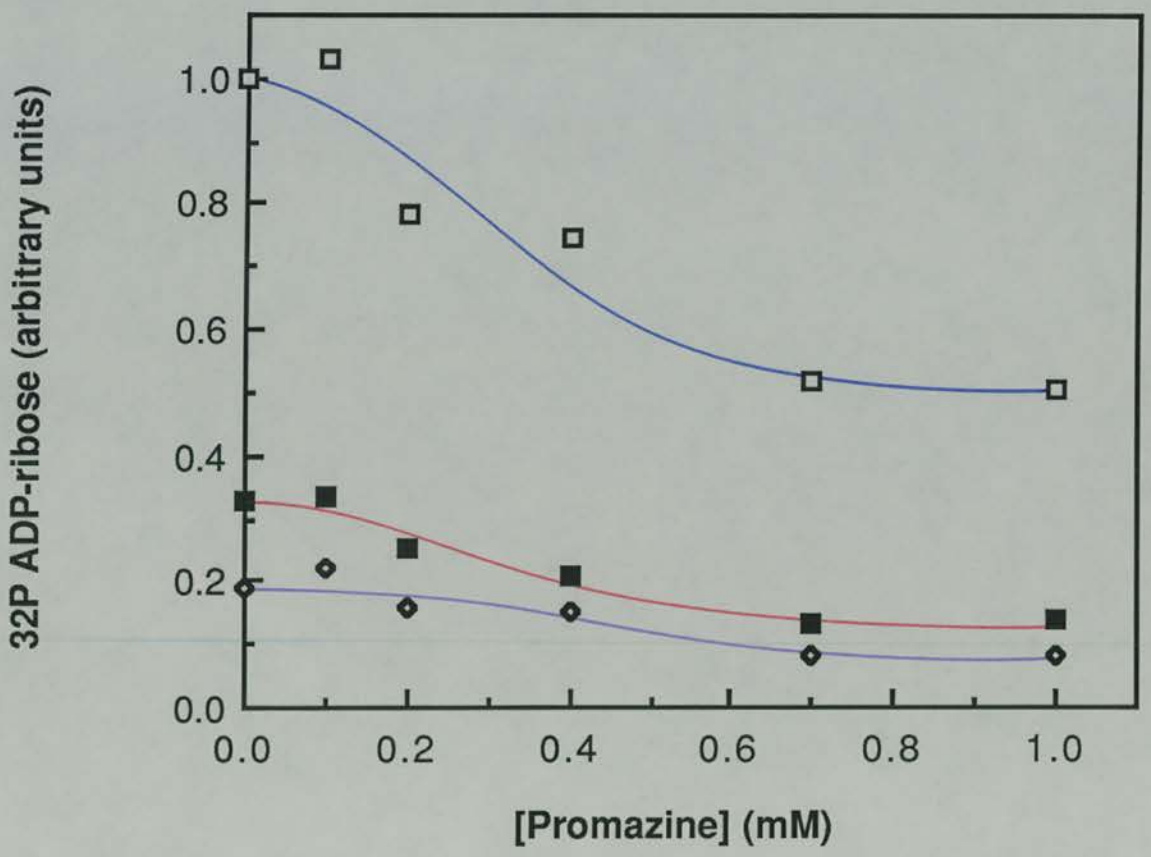
Fig. 4-7 **Effect of antidiarrhoeal drugs on the incorporation of ^{32}P-ADP-ribose into membrane proteins**

Graphs show the incorporation of ^{32}P-ADP-ribose into 40 kDa (□—□), 45 kDa (■—■), 37 kDa (◇—◇) and 66 kDa (▲—▲)proteins when mixed plasma membranes (A), **brush border membranes** (B) and basal-lateral membranes (C) were incubated with 20 μg/ml preactivated cholera toxin, 5 μM-[adenylate-^{32}P]NAD$^+$ and 0-1 mM drug for 30 min at 30°C as in section 4.4.1. The drugs used were chlorpromazine (a), amitriptyline (b), trifluoperazine (c), **promethazine (d), triflupromazine (e)** and **promazine (f)**. The arbitrary unit of 1 for mixed plasma membranes, brush border membranes and basal-lateral membranes corresponds to integrated values of 30000, 60000 and 15000 respectively, obtained from the densitometric analysis of the autoradiographs (Fig. 4-6).

B (e)


32P ADP-ribose (arbitrary units)
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.2
0.4
0.6
0.8
1.0
[Trifluprom azine] (mM)

B (f)


32P ADP-ribose (arbitrary units)
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.2
0.4
0.6
0.8
1.0
[Promazine] (mM)

C (a)

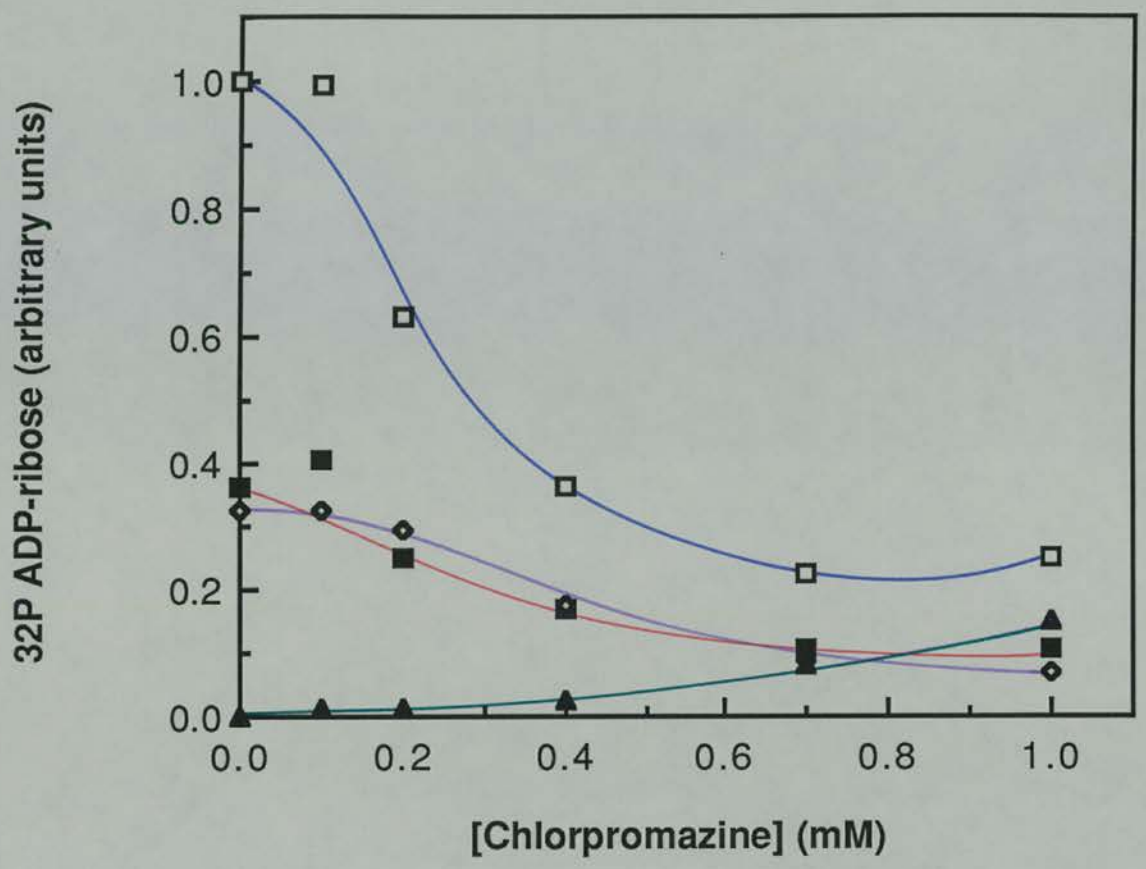
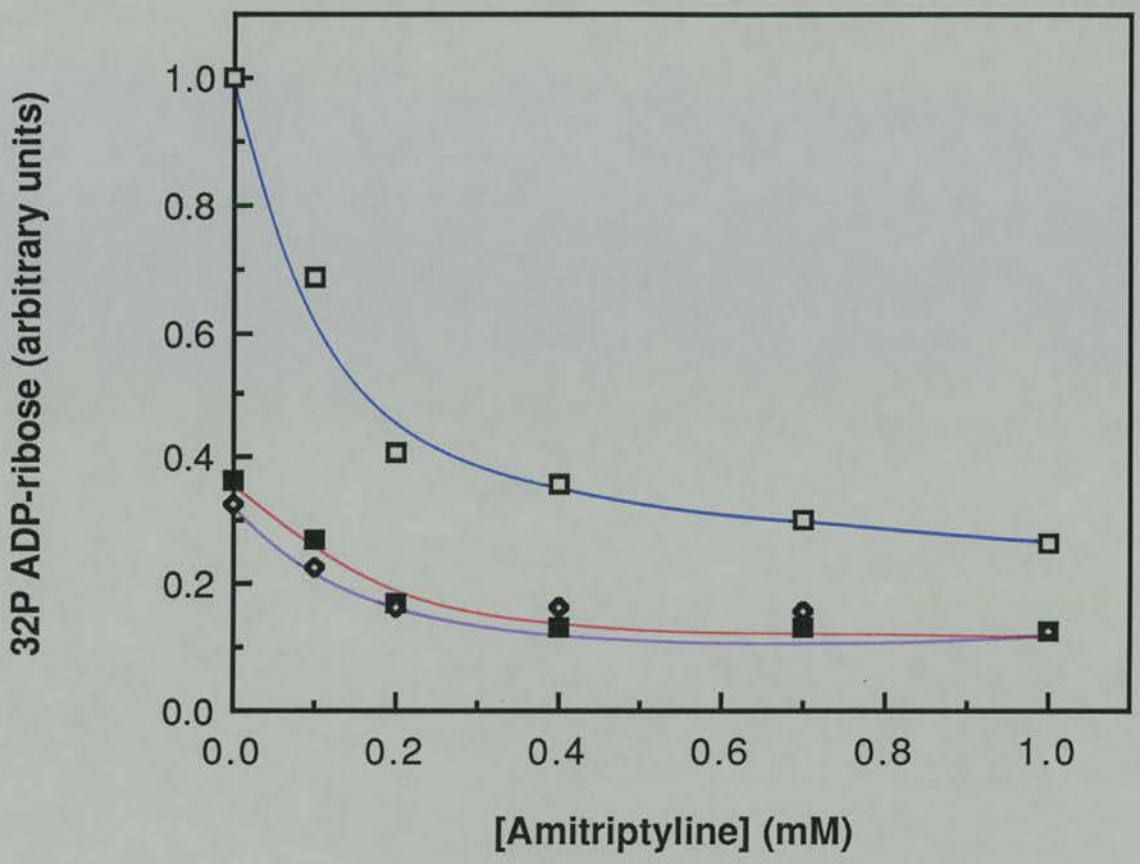


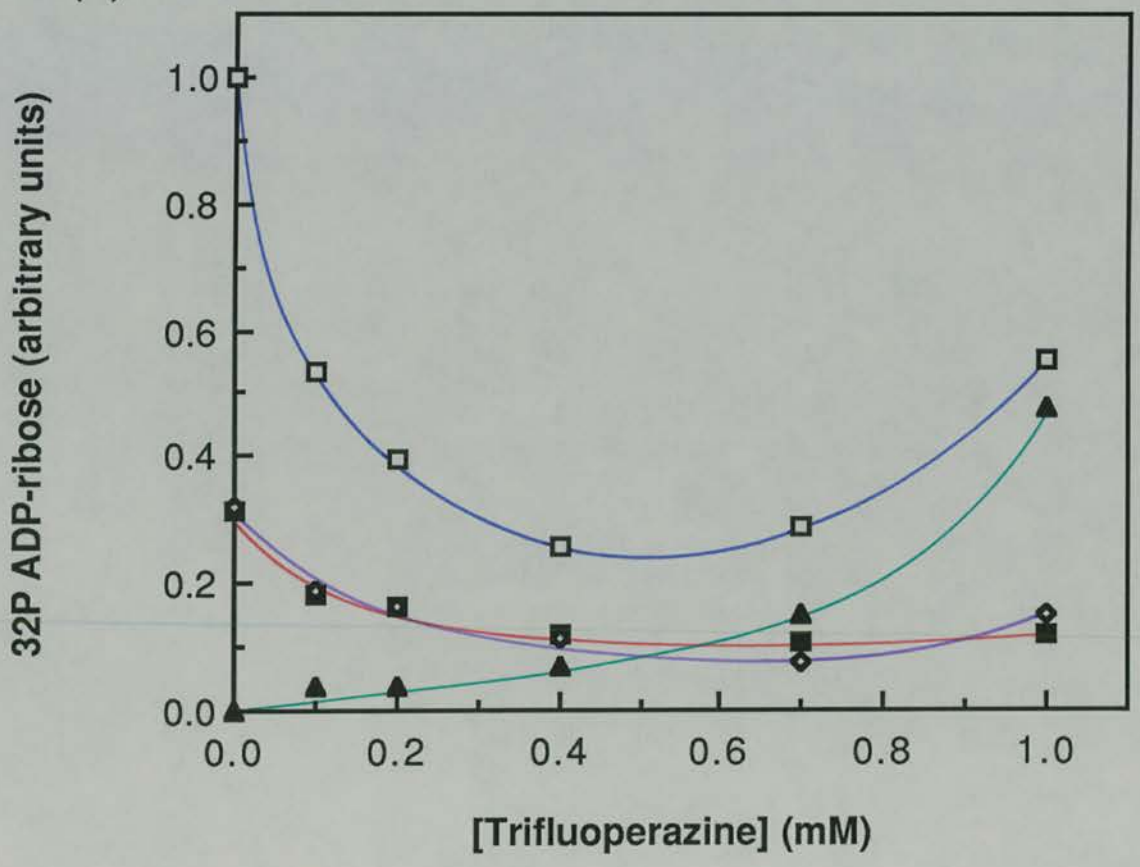
Fig. 4-7 **Effect of antidiarrhoeal drugs on the incorporation of ^{32}P-ADP-ribose into membrane proteins**

Graphs show the incorporation of ^{32}P-ADP-ribose into 40 kDa (□—□), 45 kDa (■—■), 37 kDa (◇—◇) and 66 kDa (▲—▲)proteins when mixed plasma membranes (A), brush border membranes (B) and **basal-lateral membranes (C)** were incubated with 20 μg/ml preactivated cholera toxin, 5 μM-[adenylate-^{32}P]NAD$^+$ and 0-1 mM drug for 30 min at 30°C as in section 4.4.1. The drugs used were **chlorpromazine (a), amitriptyline (b), trifluoperazine (c)**, promethazine (d), triflupromazine (e) and promazine (f). The arbitrary unit of 1 for mixed plasma membranes, brush border membranes and basal-lateral membranes corresponds to integrated values of 30000, 60000 and 15000 respectively, obtained from the densitometric analysis of the autoradiographs (Fig. 4-6).

C (b)


32P ADP-ribose (arbitrary units)
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.2
0.4
0.6
0.8
1.0
[Amitriptyline] (mM)

C (c)


32P ADP-ribose (arbitrary units)
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.2
0.4
0.6
0.8
1.0
[Trifluoperazine] (mM)

C (d)

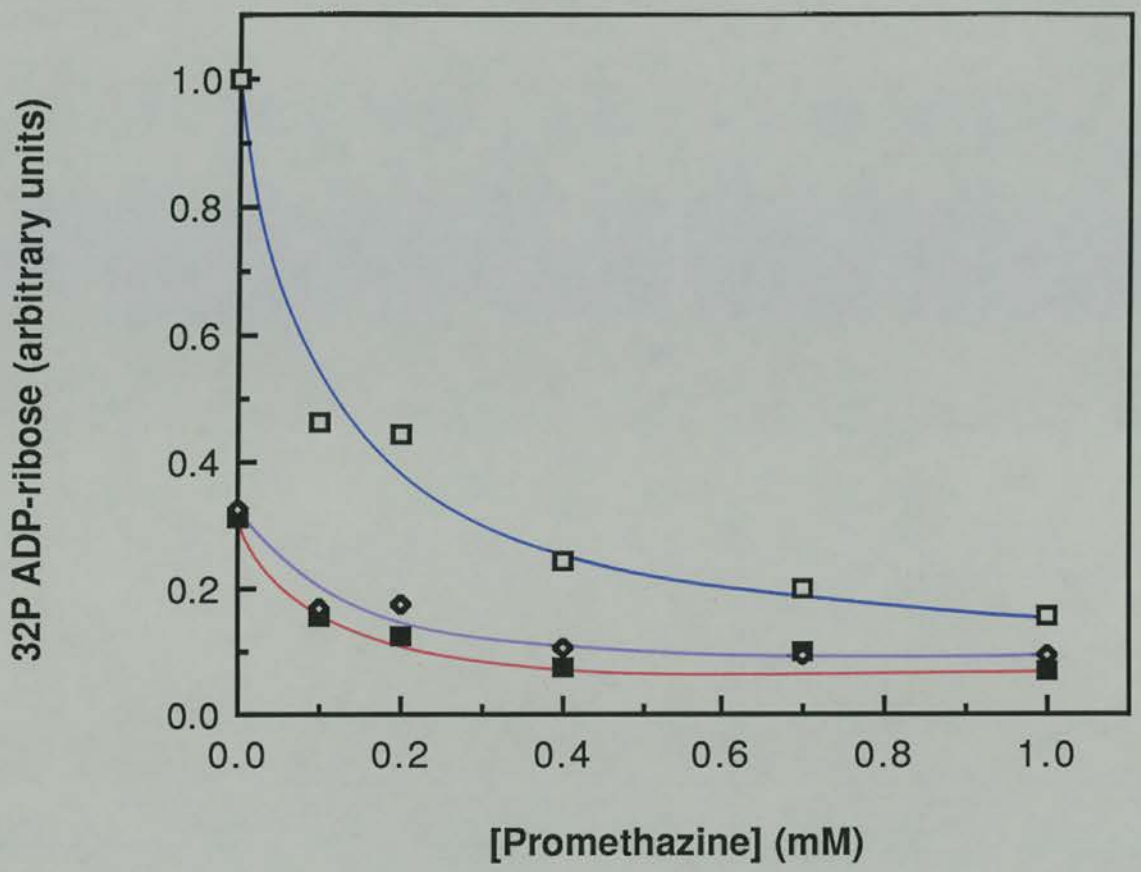
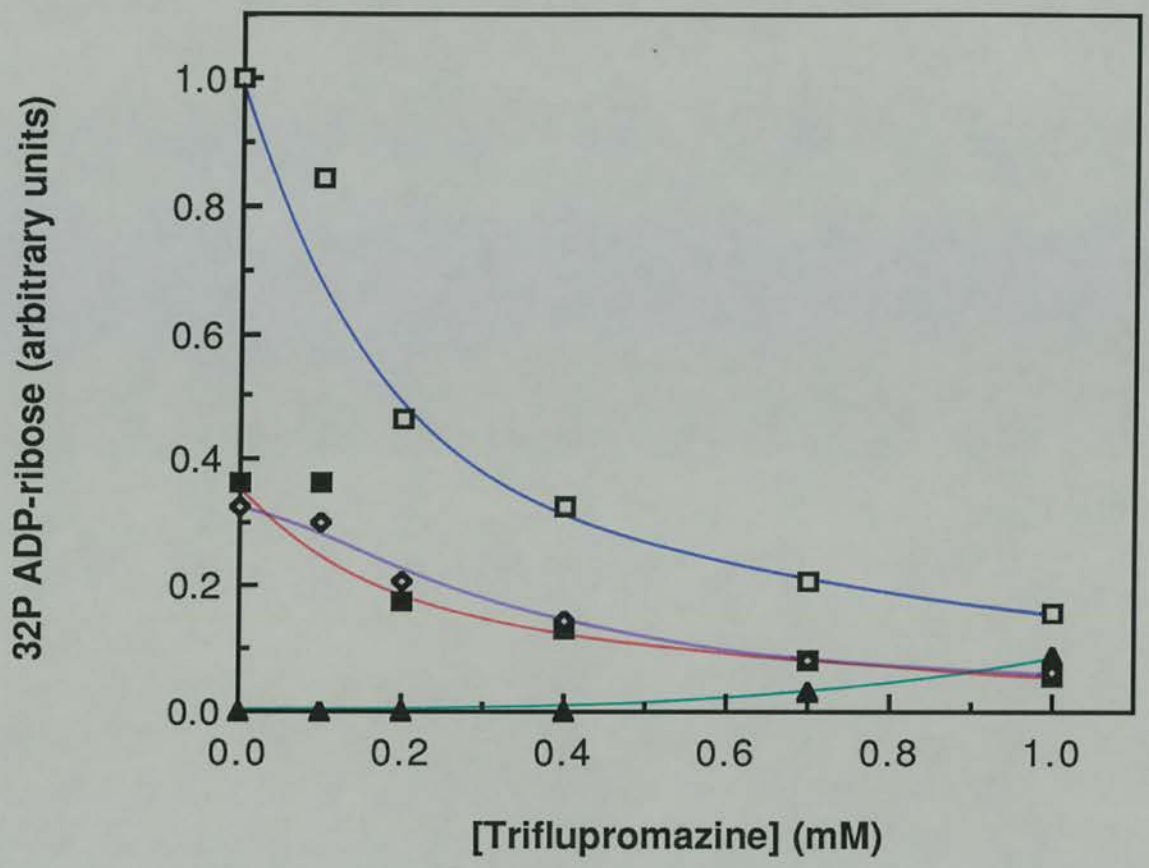


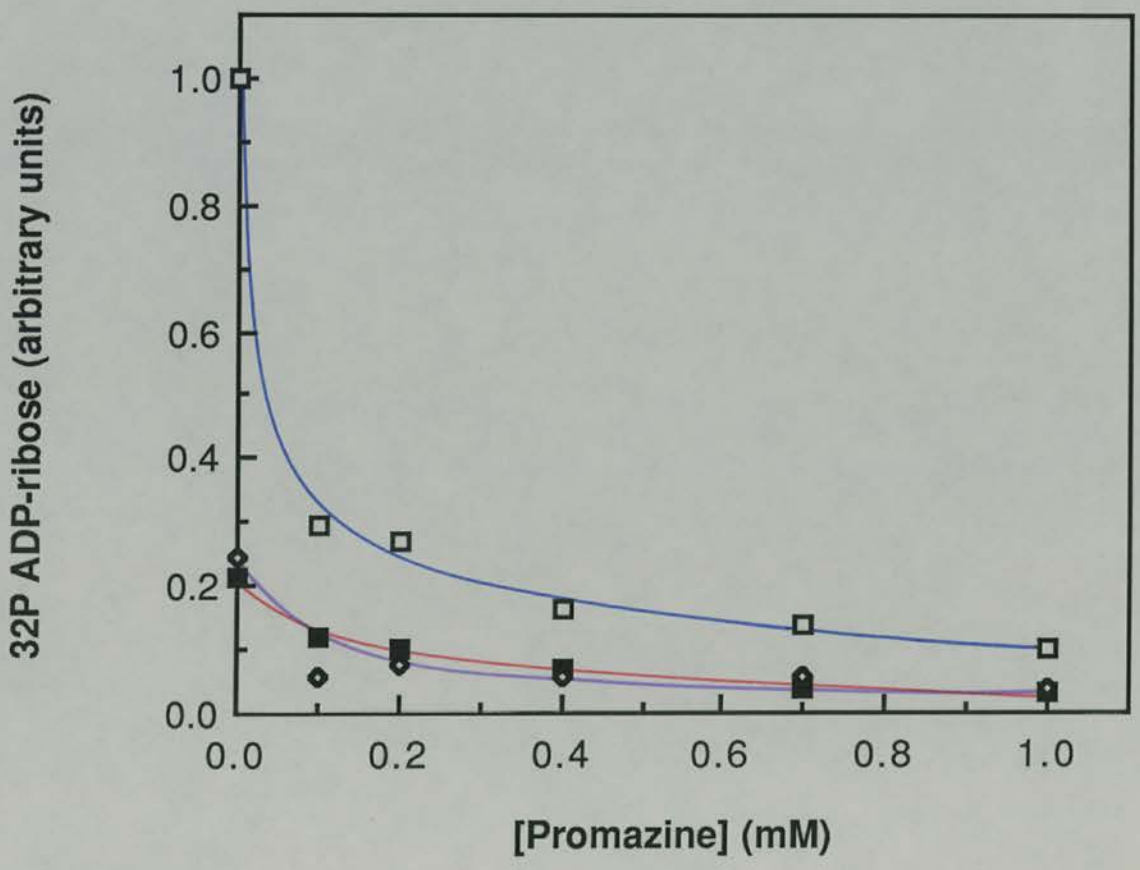
Fig. 4-7 **Effect of antidiarrhoeal drugs on the incorporation of ^{32}P-ADP-ribose into membrane proteins**

Graphs show the incorporation of ^{32}P-ADP-ribose into 40 kDa (□—□), 45 kDa (■—■), 37 kDa (◇—◇) and 66 kDa (▲—▲)proteins when mixed plasma membranes (A), brush border membranes (B) and **basal-lateral membranes (C)** were incubated with 20 μg/ml preactivated cholera toxin, 5 μM-[adenylate-^{32}P]NAD$^+$ and 0-1 mM drug for 30 min at 30°C as in section 4.4.1. The drugs used were chlorpromazine (a), amitriptyline (b), trifluoperazine (c), **promethazine (d), triflupromazine (e)** and **promazine (f)**. The arbitrary unit of 1 for mixed plasma membranes, brush border membranes and basal-lateral membranes corresponds to integrated values of 30000, 60000 and 15000 respectively, obtained from the densitometric analysis of the autoradiographs (Fig. 4-6).

C (e)


32P ADP-ribose (arbitrary units)
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.2
0.4
0.6
0.8
1.0
[Trifluprom azine] (mM)

C (f)


32P ADP-ribose (arbitrary units)
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.2
0.4
0.6
0.8
1.0
[Promazine] (mM)

Unfortunately, there was a problem with the solubility of three of the drugs, namely chlorpromazine, trifluoperazine and triflupromazine. When certain concentrations of the solubilized drugs were added to the ADP-ribosylation assay mixture they became insoluble; this was due to a rise in pH above 6.4 causing the free base to precipitate. The ADP-ribosylation assay was performed at a pH of 7.5 and no ADP-ribosylation of membrane proteins was found to occur if the pH dropped below about 7. The pH of the water-solubilized drugs was about pH 4-5, but the buffering capacity of the ADP-ribosylation assay mixture was sufficient to ensure that when the drug solutions were added to the reaction mixture the pH did not drop much, if at all, below 7.5. However, this increase in the pH to 7.5 did cause the precipitation of the free base and since this pH was necessary for the ADP-ribosylation to take place, it was an unsolvable problem. It is also possible that these drugs, at concentrations at which they become less soluble, cause the precipitation of other proteins in the reaction mixture. Therefore, it is difficult to be certain as to the extent of insolubility at the higher drug concentrations. Generally, this solubility became noticeable at final drug concentrations of 0.7 and 1 mM, although with trifluoperazine it also occurred at a concentration of 0.4 mM. This insolubility has resulted in two effects, one of which was an apparent increase in protein content, for mainly the proteins of 66 and 40 kDa, on the gels (Fig. 4-6 I(a), III(a) and V(a) for mixed plasma (A), brush border (B) and basal-lateral (C) membranes). The increase in the protein content of these

proteins, at concentrations of 0.7 and 1 mM for chlorpromazine and trifluprομazine, and at concentrations of 0.4, 0.7 and 1 mM for trifluoperazine, has resulted in the second effect, which was an apparent increase in the ADP-ribosylation of these proteins at these drug concentrations (Fig. 4-7). This second effect was particularly noticeable with the drug trifluoperazine (Fig. 4-7B(c) and C(c)), and this was the drug which seemed to be the most insoluble at the concentrations mentioned above (Fig. 4-6 III(a) for A, B and C). These two effects are probably due to the drugs, originating from the cytosolic fraction S_4 (see Fig. 2-1) having become insoluble, binding to the soluble proteins and pelleting them with the membranes when centrifuged at 9,000g. This would then explain the appearance of the 66 kDa protein on the gels at the higher concentrations, which as discussed in section 4.3.2 is bovine serum albumin. Due to this insolubility problem it was impossible to say what the true concentrations of these drugs were in the assay mixtures, and so it is difficult to make any comparison between the effects of the drugs on each membrane fraction. However, apart from this problem there did not seem to be any consistency in the effect of certain drugs on one membrane fraction and their effects on one of the other fractions, even if comparisons are made at the lower drug concentrations where there appeared to be no insolubility problem. For example, with mixed plasma membranes the greatest inhibition of incorporation of ^{32}P-ADP-ribose into the 40 kDa protein was achieved with chlorpromazine (Fig. 4-7A(a)) and trifluoperazine (Fig. 4-7A(c)) and the worst inhibition was achieved with promazine (Fig. 4-7A(f)), but

on the other hand with basal-lateral membranes by far the largest inhibition was caused by promazine (Fig. 4-7C(f)). Although, for mixed plasma membranes there does appear to be a greater initial inhibition of ADP-ribosylation by chlorpromazine (Fig. 4-7A(a)), trifluoperazine (Fig. 4-7A (c)) and triflupromazine (Fig. 4-7A(e)), than by amitriptyline (Fig. 4-7A(b)), promethazine (Fig. 4-7A(d)), and promazine (Fig. 4-7A(f)). This may also be the case for brush border membranes (Fig. 4-7B(a)-(f)), but it is not as obvious as for mixed plasma membranes. Another striking effect was the much smaller percentage inhibition of the incorporation of ^{32}P-ADP-ribose on the 40 kDa protein occuring in brush border membranes (40-50%) (Fig. 4-7B) compared to mixed plasma membranes (80%) (Fig. 4-7A) and basal-lateral membranes (80%) (Fig. 4-7C). No immediate ideas come to mind explaining the reason for this, except that it may be indicative of there being more than one site of incorporation of ^{32}P-ADP-ribose on the protein, and that one of these sites is affected to a much greater extent by the drugs than the other(s).

It is therefore difficult to draw any conclusions from this work, particularly as to a relationship between the inhibition of ADP-ribosylation with the known antidiarrhoeal and anticalmodulin activities of the drugs. All that can be stated is that there is obviously some inhibition occuring and a lot more work needs to be carried out to try and resolve the many problems that have been found to occur. Deducing the mechanism by which the drugs inhibit ADP-

ribosylation is of major importance. Since the drugs are known to bind calmodulin, does this mean that calmodulin is also needed for the ADP-ribosyltransferase activity of the active A_1 peptide of cholera toxin? This may be more than a possibility, but it does seem strange that a bacterium produces a toxin that is dependent on a molecule of its host for its activity. Although, the bacteria *Bordetella pertussis* and *Bacillus anthracis*, responsible for the diseases whooping cough and anthrax respectively, secrete adenylate cyclase, and adenylate cyclase is known to require the eukaryotic protein calmodulin for its activity (Amiranoff *et al.*, 1983). Also, as mentioned in section 1.4.2, it has been shown that the active A_1 peptide of cholera toxin requires several eukaryotic (Kahn *et al.*, 1988) ADP-ribosylation factors, which are all GTPases, for its activity (Tsai *et al.*, 1987, 1988; Price *et al.*, 1988), and thus is dependent on its host for its activity.

CHAPTER FIVE

ACTIVATION OF ADENYLATE CYCLASE

5.1 Introduction

As discussed previously in sections 1.6 and 4.1, it has never been clearly shown how cholera toxin can get to its substrate in intestinal cells to cause the chain reaction of events resulting in the characteristic diarrhoea of this disease. The toxin is produced in the lumen of the gut by *V. cholerae*, and enters the cell at the brush border membrane where it ADP-ribosylates the GTP-activated G protein causing the release of $G_{s\alpha}$ (the α subunit of the stimulatory G protein) into the cell cytosol (Chapter Four). This $G_{s\alpha}$ must then move through the cytosol until it comes into contact with, and causes the activation of, the catalytic component of adenylate cyclase resulting in the increase in production of cyclic AMP from ATP. This catalytic component is generally thought to be located only on the opposite side of the cell from the point of entry of the toxin, i.e. exclusively in the basal-lateral membrane (Murer *et al.*, 1976; Walling *et al.*, 1978). Therefore, it seemed necessary to investigate the cholera toxin activation of the cyclase in all three membrane fractions (mixed plasma, brush border and basal-lateral) for the possibility of a cholera-toxin-activatable adenylate cyclase existing in the brush border as well as in the basal-lateral membrane.

In another set of experiments, the six antidiarrhoeal drugs shown in Fig. 4-1 were tested for their ability to inhibit the cholera-toxin-induced activation of adenylate cyclase. This was done to try and deduce a relationship between the inhibition of the cyclase with the anticalmodulin and

antidiarrhoeal activities of the drugs.

5.2 Activation of adenylate cyclase by cholera toxin

5.2.1 Method

The adenylate cyclase assay measures the activity of the cyclase by its ability to convert [α-^{32}P]ATP to cyclic [^{32}P]AMP. Details of the calculation involved in determining the amount of cyclic AMP produced per min per mg protein are shown in Appendix C.

Prior to investigating the time-dependent activation of the cyclase by both cholera toxin and sodium fluoride, it was necessary to determine the concentration of toxin required to achieve the maximal activation of the enzyme, and the amount of membrane protein needed to get activation. For determining the concentration of cholera toxin needed, basically, [α-^{32}P]ATP and 50 μg ADP-ribosylated membrane protein were incubated in a reaction volume of 0.1 ml for 20 min at 30°C as outlined in section 2.2.3.1: membrane proteins were ADP-ribosylated by 0.001-50 μg/ml preactivated cholera toxin as outlined in section 2.2.2.1. This assumed that the 20 min incubation period and the amount of membrane protein used for each fraction corresponded to a point on the linear portion of the respective time course and dose response curves. For determining the amount of membrane protein needed, similarly, [α-^{32}P]ATP and 0-100 μg ADP-ribosylated membrane protein were incubated in a reaction volume of 0.1 ml for 20 min at 30°C as outlined in section

2.2.3.1: membrane proteins were ADP-ribosylated by 20 μg/ml preactivated cholera toxin as outlined in section 2.2.2.1. This assumed that the 20 min incubation period and the concentration of the toxin used were suitable conditions for the assay. To investigate the time-dependent activation of adenylate cyclase in each membrane fraction by both cholera toxin and sodium fluoride, [α-^{32}P]ATP and 50 μg ADP-ribosylated membrane protein were incubated in a reaction volume of 0.1 ml, at 30°C for 0-60 min as outlined in section 2.2.3.1: membrane proteins were ADP-ribosylated by 20 μg/ml preactivated cholera toxin as outlined in section 2.2.2.1.

5.2.2 Results and discussion

Cholera toxin activated intestinal adenylate cyclase at concentrations above 0.01 μg/ml, the maximal effect being at about 10 μg/ml (Fig. 5-1). Sodium fluoride activated maximally at about 10 mM. Therefore, the toxin concentration of 20 μg/ml used in the assays was found to be suitable to achieve maximal activation. The membrane protein dose response curve, shown in Figure 5-2, quite clearly shows that there was a linear response up to at least 100 μg protein. Therefore, the 50 μg of membrane protein routinely used in the assay was found to be enough to achieve good activation of the cyclase.

The results of the time courses for the activation of adenylate cyclase in mixed plasma, basal-lateral and brush border membranes by both cholera toxin and sodium fluoride

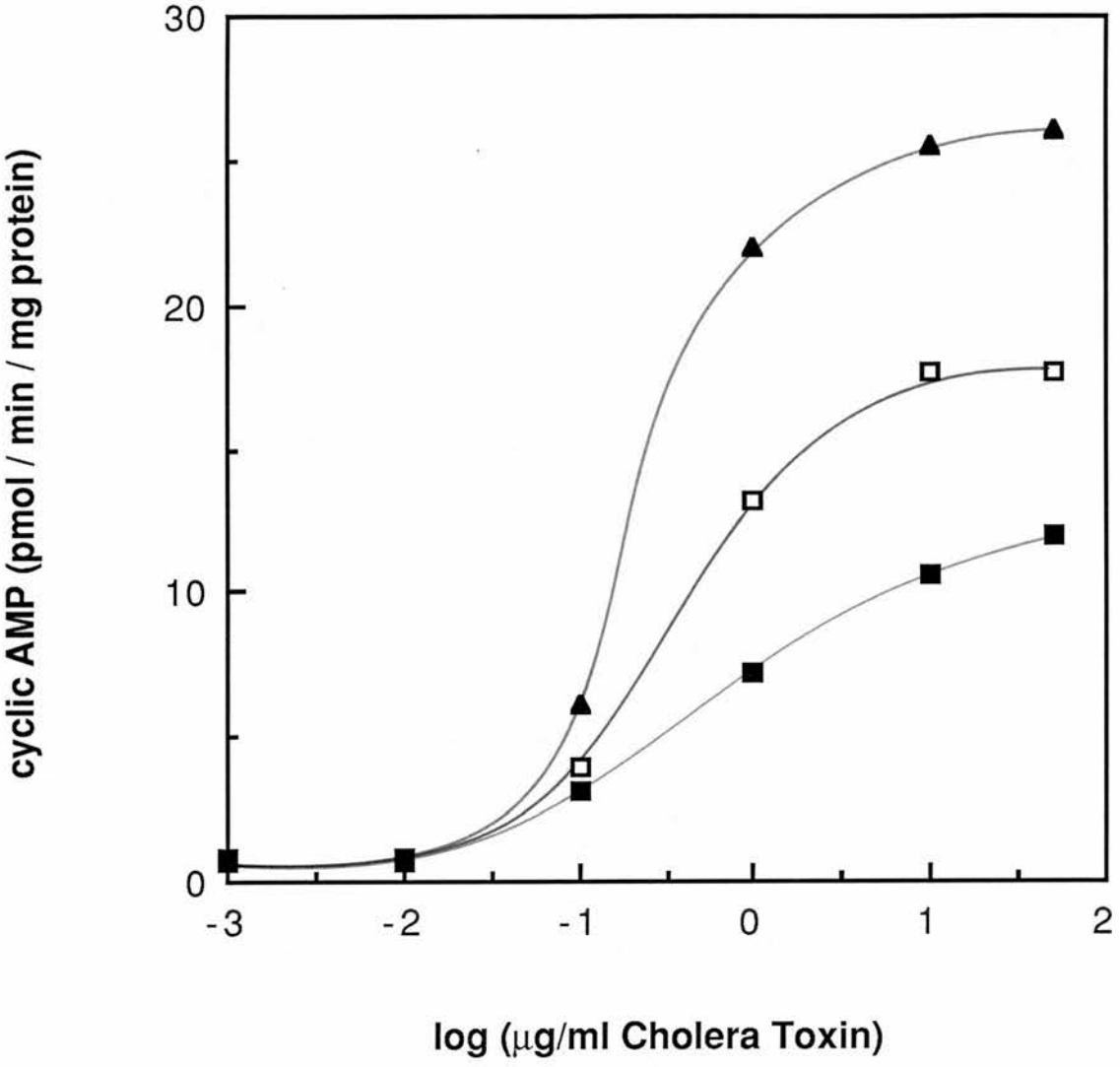


Fig. 5-1 Effect of cholera toxin concentration on the activation of adenylate cyclase.

Mixed plasma membranes (□—□), basal-lateral membranes (▲—▲) and brush border membranes (■—■) were incubated with 0.001-50 μg/ml preactivated cholera toxin and [α-^{32}P]ATP for 20 min at 30°C as in section 5.2.1.

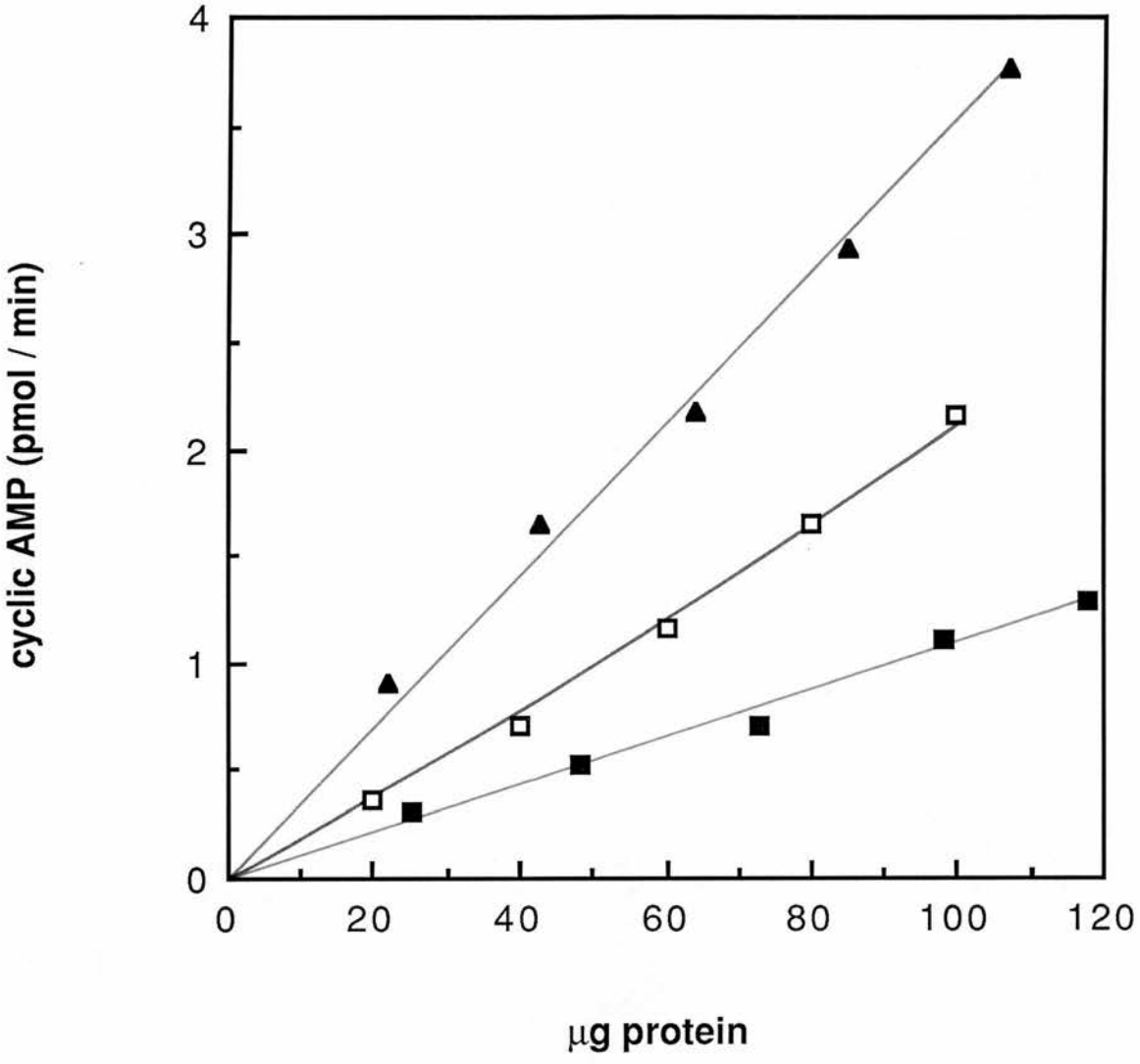


Fig. 5-2 Effect of amount of protein on the activation of adenylate cyclase by cholera toxin

Mixed plasma membranes (□—□), basal-lateral membranes (▲—▲) and brush border membranes (■—■) were incubated with 20 µg/ml preactivated cholera toxin and [α-^{32}P]ATP for 20 min at 30°C as in section 5.2.1.

are shown in Figure 5-3. The graphs show that there was a greater activation of the cyclase in the basal-lateral membrane fraction compared to the mixed plasma membrane fraction for both the toxin and the fluoride, and there was greater activity in the mixed plasma compared to the brush border membrane fraction. Table 5-1 shows the specific activities of the enzyme in the three fractions after activation by toxin and fluoride: the values were calculated from the linear portions of the time courses shown in Figure 5-3. The relative specific activities (Table 5-1) show a 2.7- and 2.3-fold increase in adenylate cyclase activity by cholera toxin and sodium fluoride respectively in basal-lateral membranes compared to brush border membranes. This is analagous to the enrichment of (Na^+-K^+)ATPase in these fractions (Table 3-2): 2.6-fold greater in basal-lateral membranes than in brush border membranes. The basal level of adenylate cyclase activity was found to be very low in each of the fractions: less than 1 pmol cyclic AMP produced per min per mg membrane protein. These results imply that the activation of adenylate cyclase in the brush border membrane is due to contamination by basal-lateral membranes and, therefore, the catalytic subunit of the cyclase is present only in basal-lateral membranes. The graphs (Fig. 5-3) show that the distribution of activities in the three fractions for the toxin and the fluoride are almost identical, implying that the two effectors activate the cyclase either via the same protein or via different proteins in the same fraction. For this reason, the effect of sodium fluoride on membranes that had been previously

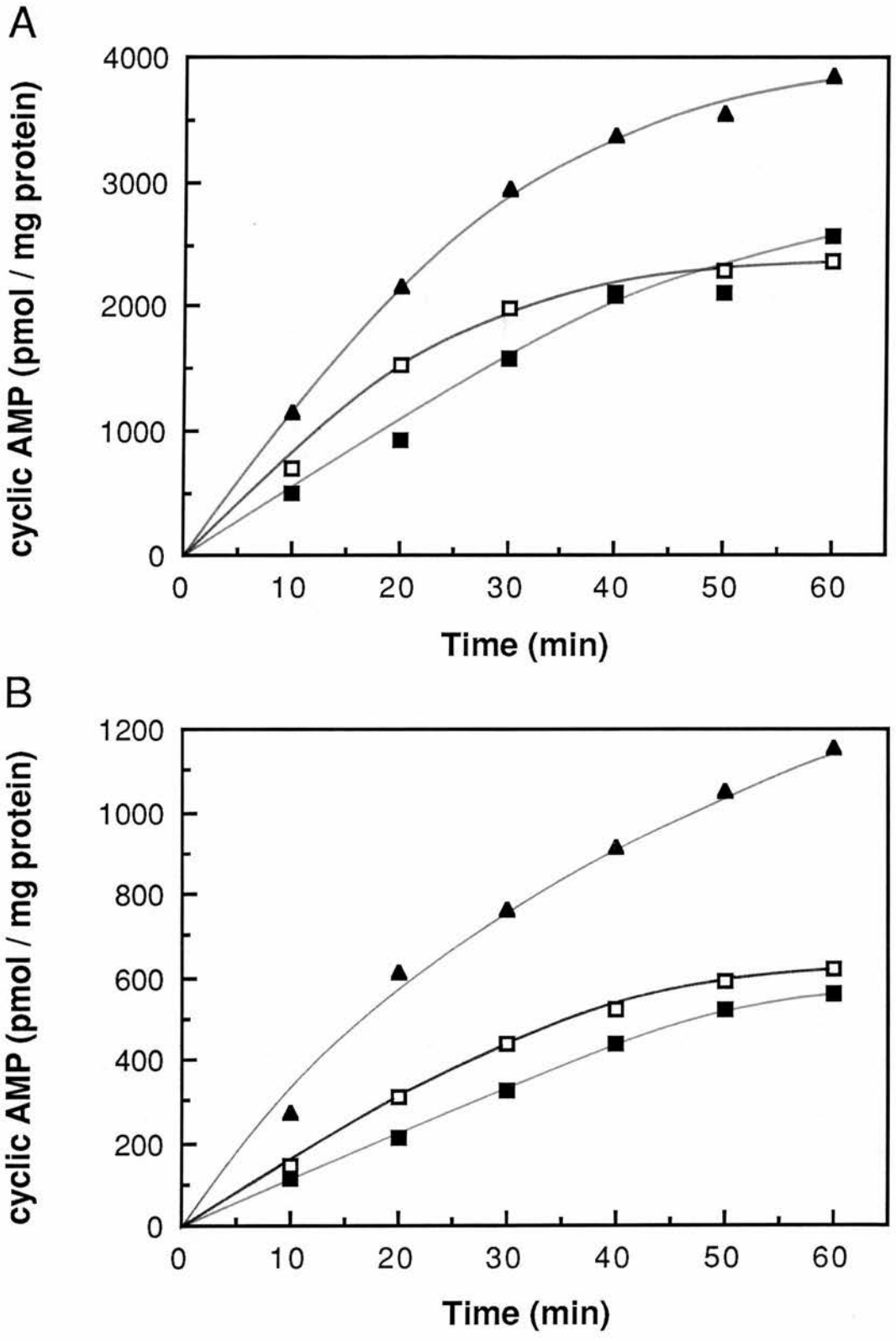


Fig. 5-3 **Activation of adenylate cyclase by sodium fluoride and cholera toxin.**

Mixed plasma membranes (□—□), basal-lateral membranes (▲—▲) and brush border membranes (■—■) were incubated with either (A) 10 mM sodium fluoride or (B) 20 μg/ml preactivated cholera toxin, and [α-^{32}P]ATP at 30°C as in section 5.2.1.

Table 5-1 Activation of adenylate cyclase in the membrane fractions

MPM = Mixed Plasma Membranes, BLM = Basal-Lateral Membranes and BBM = Brush Border Membranes. S.A.* = Specific Activity in pmol cyclic AMP produced.min^{-1}.(mg protein)$^{-1}$. Basal activity values are expressed as pmol cyclic AMP produced.min^{-1}.(mg protein)$^{-1}$. R.S.A.+ = Specific Activity Relative to the mixed plasma membranes. For experimental details of the adenylate cyclase assay see section 2.2.3.1.

Fraction	Adenylate cyclase basal activity	Adenylate cyclase activation by NaF (10 mM)		Adenylate cyclase activation by Cholera Toxin (20 μg/ml)	
		S.A.*	R.S.A.+	S.A.	R.S.A.
MPM	<1	74	1.0	15	1.0
BLM	<1	115	1.6	29	1.9
BBM	<1	53	0.7	11	0.7

ADP-ribosylated by cholera toxin was investigated.

5.3 Effect of sodium fluoride on ADP-ribosylated membranes

5.3.1 Method

ADP-ribosylated membranes (mixed plasma, basal-lateral and brush border) (see section 2.2.2.1) were incubated in a reaction volume of 0.1 ml, with [α-^{32}P]ATP and sodium fluoride for 20 min at 30°C as outlined in section 2.2.3.1: membrane proteins were ADP-ribosylated with 20 μg/ml preactivated cholera toxin as outlined in section 2.2.2.1. The resulting activation of adenylate cyclase was compared to that produced by membranes treated with either cholera toxin or sodium fluoride alone. When looking at the effect of cholera toxin on the activation of adenylate cyclase, membranes were ADP-ribosylated prior to the adenylate cyclase assay for 30 min at 30°C, whereas when sodium fluoride was used as the activator the membranes were preincubated for 30 min at 30°C. This was to ensure that, prior to the adenylate cyclase assay, all membranes had been previously incubated for the same length of time and so the cyclase would have been subject to the same degree of inactivation.

5.3.2 Results and discussion

The results of the experiment are shown in Table 5-2. If sodium fluoride activated the cyclase via a different regulatory component from that of cholera toxin, then the amount of cyclic AMP produced would be expected to be the

Table 5-2 Effect of sodium fluoride on membranes pretreated with cholera toxin

The experimental details of the adenylate cyclase assay are as in section 2.2.3.1. Incubation was for 20 min at 30°C. MPM = Mixed Plasma Membranes, BLM = Basal-Lateral Membranes and BBM = Brush Border Membranes. S.A.* = Specific Activity in pmol cyclic AMP produced.min^{-1}.(mg protein)$^{-1}$. R.S.A.+ = Specific Activity Relative to the mixed plasma membranes.

Fraction	Adenylate cyclase activation by					
	NaF (10 mM)		Cholera Toxin (20 μg/ml)		NaF (10 mM) in Cholera Toxin (20 μg/ml) pretreated membranes	
	S.A.*	R.S.A.+	S.A.	R.S.A.	S.A.	R.S.A.
MPM	52	1.0	28	1.0	23	1.0
BLM	109	2.1	38	1.4	28	1.2
BBM	50	1.0	26	0.9	20	0.9

additive total produced by each effector on its own, i.e. for basal-lateral membranes a value of 147 pmol cyclic AMP produced per minute per mg membrane protein would be expected. If, on the other hand, sodium fluoride activated the same protein as cholera toxin, then it is possible that once $G_{s\alpha}$ has been modified by the toxin it cannot be activated by fluoride ions, and so the result would be that only the effect of the toxin would be seen. The results of the experiment (Table 5-2) appear to agree with this latter possibility. Therefore, it is quite clear that sodium fluoride does not increase the specific activity of adenylate cyclase after the regulatory component, $G_{s\alpha}$, has been modified by cholera-toxin-induced ADP-ribosylation. This result is consistent with what is generally accepted, which is that fluoride ions activate adenylate cyclase, as does cholera toxin, by stimulating the regulatory component, $G_{s\alpha}$. Hence, as well as the regulatory protein being known as the GTP-binding protein, the hormone-stimulated GTPase, the N protein (because it binds nucleotides) and the G protein (because it binds guanine nucleotides), it is also known as the G/F protein since it confers responsiveness to both guanine nucleotides and fluoride.

5.4 Effect of antidiarrhoeal drugs on the activation of adenylate cyclase by cholera toxin

5.4.1 Method

ADP-ribosylated membranes (mixed plasma, basal-lateral and brush border) (see section 2.2.2.1) were incubated in a reaction volume of 0.1 ml, with [α-^{32}P]ATP and 0-3 mM final concentration of each of the drugs shown in Figure 4-1, for 20 min at 30°C as outlined in section 2.2.3.1: membrane proteins were ADP-ribosylated with 20 μg/ml preactivated cholera toxin as outlined in section 2.2.2.1. All drugs were freshly prepared for each experiment, as a 15 mM stock solution, by solubilization in double-distilled water because they were very light sensitive, and they oxidized on prolonged exposure to air.

5.4.2 Results and discussion

As discussed in sections 1.4.3. and 1.4.4, there was good reason to believe that the phenothiazines would inhibit adenylate cyclase activity, since the drugs are known to bind calmodulin (Prozialeck and Weiss, 1982) and calmodulin is known to be involved in the activation of adenylate cyclase (Amiranoff *et al.*, 1983).

The results of the experiments are shown in Figure 5-4. As in section 4.4, the effects of the six drugs on each membrane fraction were investigated separately. Therefore, the quantitative inhibition of cyclic AMP production in each membrane fraction for a particular drug cannot be compared, but the trends can be. However, this does not matter since

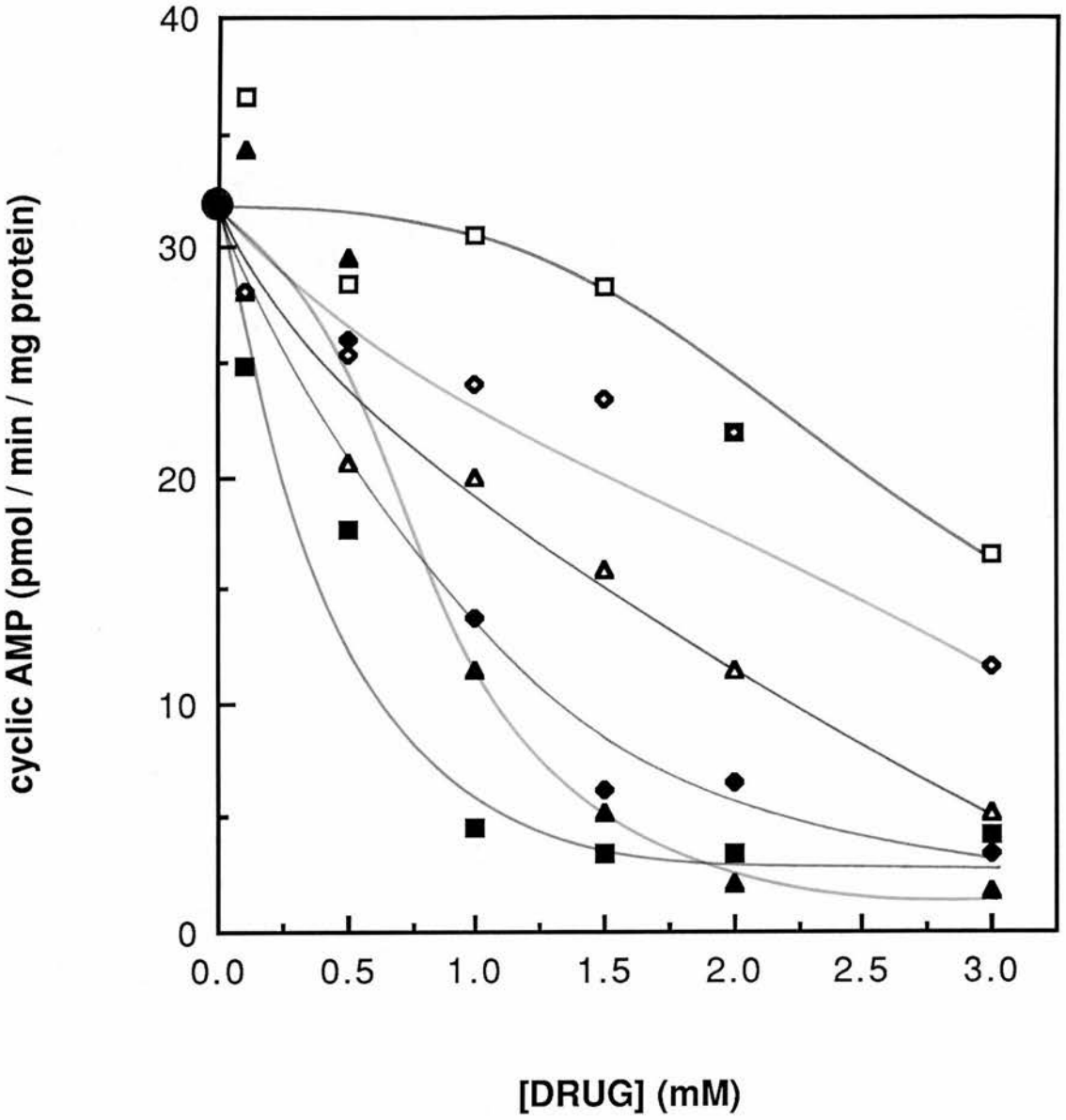


Fig. 5-4 Effect of antidiarrhoeal drugs on the activation of adenylate cyclase by cholera toxin.

Mixed plasma membranes (A), basal-lateral membranes (B) and brush border membranes (C) were incubated with 20 µg/ml preactivated cholera toxin, [α-^{32}P]ATP and 0-3 mM drug for 20 min at 30°C as in section 5.4.1. The drugs used were chlorpromazine (▲—▲), amitriptyline (△—△), trifluoperazine (■—■), promethazine (□—□), triflupromazine (◆—◆) and promazine (◇—◇).

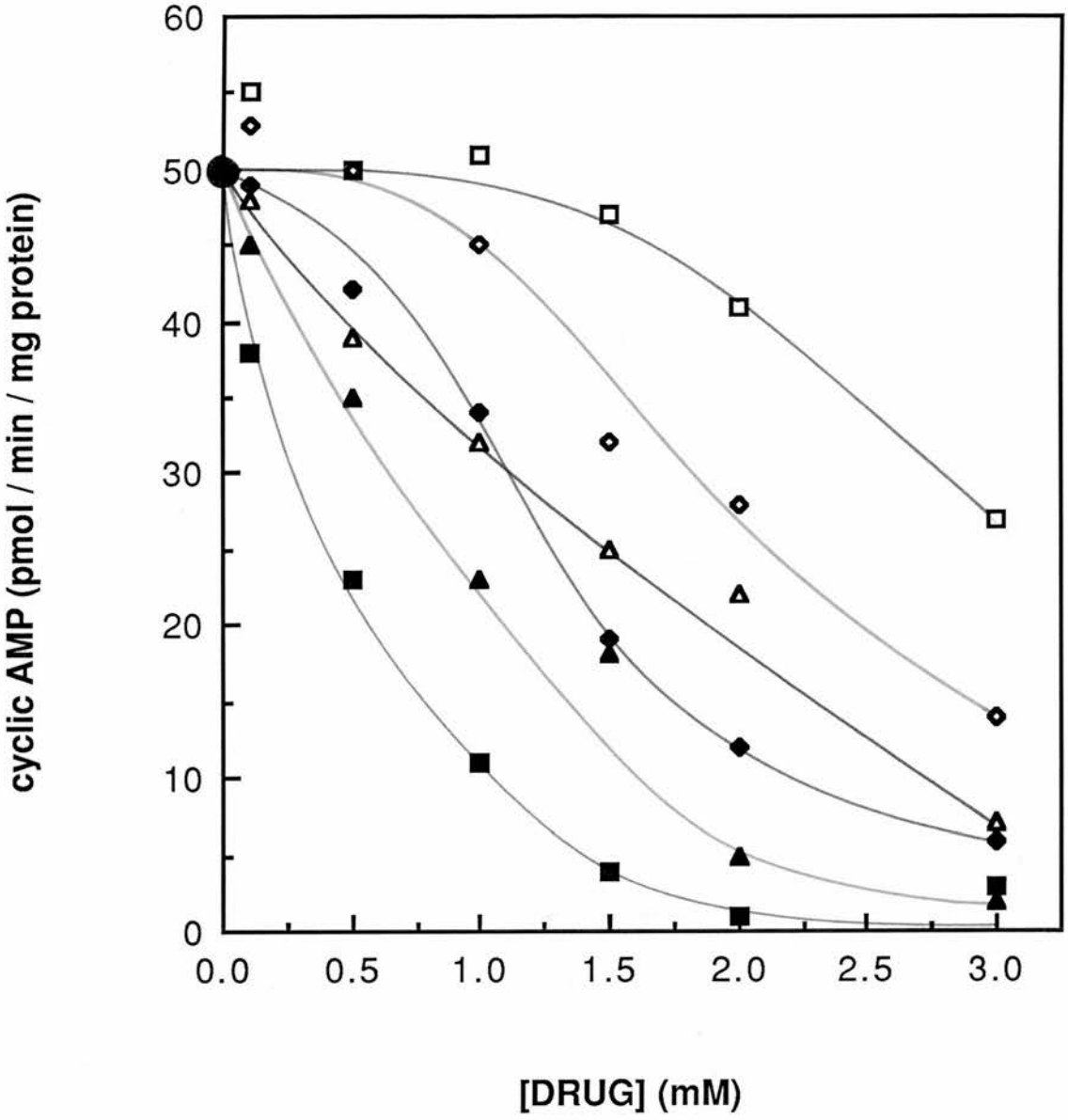


Fig. 5-4 **Effect of antidiarrhoeal drugs on the activation of adenylate cyclase by cholera toxin.**

Mixed plasma membranes (A), **basal-lateral membranes (B)** and brush border membranes (C) were incubated with 20 μg/ml preactivated cholera toxin, [α-^{32}P]ATP and 0-3 mM drug for 20 min at 30°C as in section 5.4.1. The drugs used were chlorpromazine (▲—▲), amitriptyline (△—△), trifluoperazine (■—■), promethazine (□—□), triflupromazine (◆—◆) and promazine (◇—◇).

C

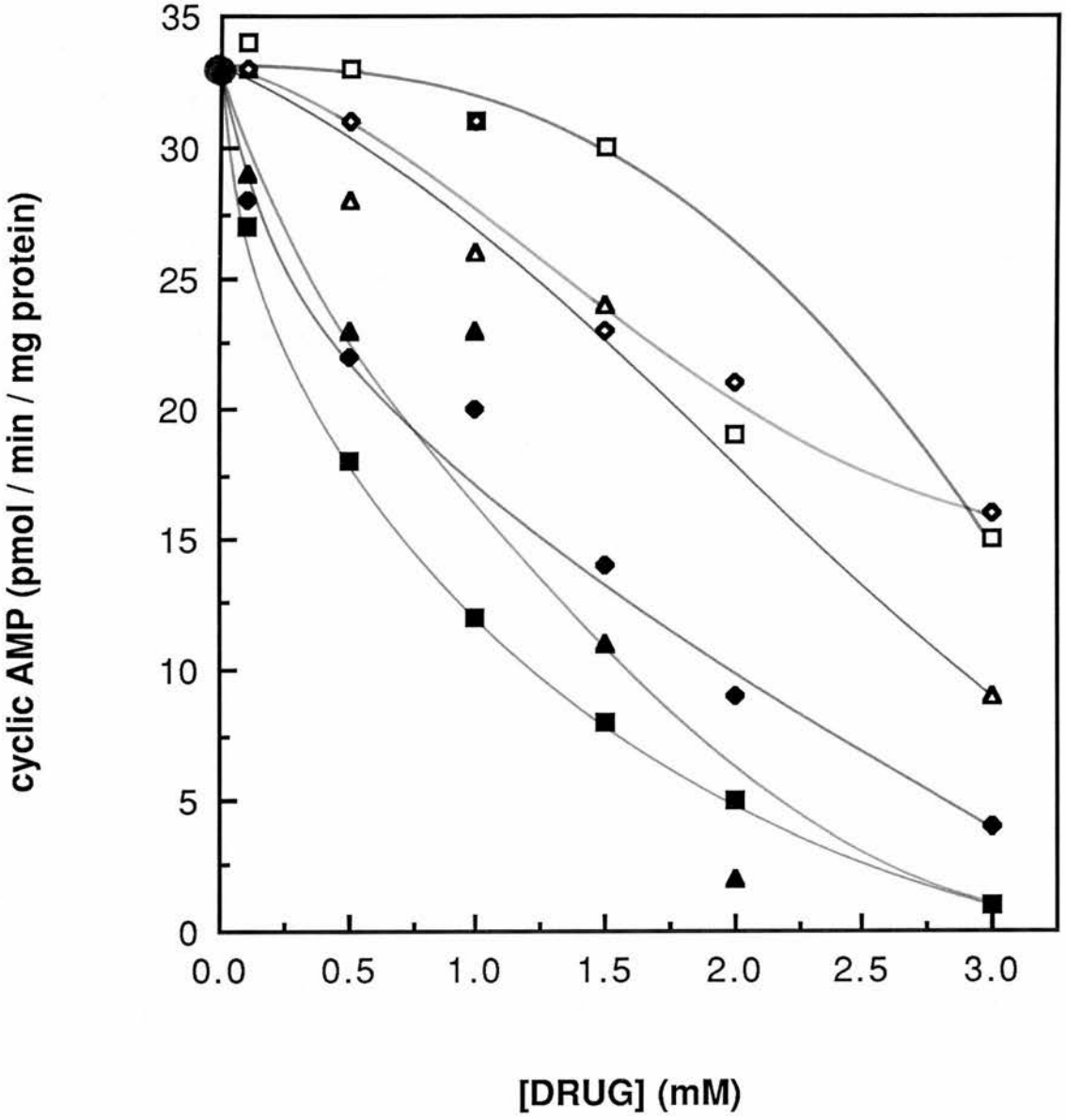


Fig. 5-4 **Effect of antidiarrhoeal drugs on the activation of adenylate cyclase by cholera toxin.**

Mixed plasma membranes (A), basal-lateral membranes (B) and **brush border membranes (C)** were incubated with 20 μg/ml preactivated cholera toxin, [α-^{32}P]ATP and 0-3 mM drug for 20 min at 30°C as in section 5.4.1. The drugs used were chlorpromazine (▲—▲), amitriptyline (△—△), trifluoperazine (■—■), promethazine (□—□), triflupromazine (◆—◆) and promazine (◇—◇).

quantitative comparisons of the amount of cyclic AMP produced in each fraction have been previously measured in section 5.2 (Fig. 5-3 and Table 5-1).

There was a similar problem with the solubility of some of the drugs at certain concentrations when added to the adenylate cyclase reaction mixture, as occurred when the effects of the drugs on ADP-ribosylation were investigated in section 4.4. The drugs which became insoluble were again chlorpromazine, trifluoperazine and triflupromazine, but in this case the drugs appeared to come out of solution at the slightly higher concentrations of 1 or 1.5 mM and greater. The drugs probably became insoluble at these higher concentrations because of a difference in the ionic strength of this reaction mixture, which is a combination of the ADP-ribosylation and adenylate cyclase assay reaction mixtures, with that of the ADP-ribosylation assay reaction mixture alone described in section 4.4. The insolubility was once again due to the pH of the reaction mixture being 7.5. However, as expected the drugs do appear to have continued inhibiting the cyclase at these concentrations (see Fig. 5-4A, B and C), since not all of the drug can have become insoluble at these concentrations and pH: the insoluble precipitate observed was probably a combination of the insoluble drug and some other component of the reaction mixture which was also becoming insoluble, as was the case in section 4.4. Although these drugs appeared to become insoluble at these higher concentrations, it seems that with mixed plasma membranes (Fig. 5-4A) maximal inhibition was

still achieved with chlorpromazine, trifluoperazine and triflupromazine at these concentrations. With basal-lateral membranes and brush border membranes maximal inhibition occured at about 2 mM (Fig. 5-4B) and 2-3 mM (Fig. 5-4C) respectively. However, unlike the results of the previous chapter where no firm conclusions could be drawn as to an effect of the drugs on ADP-ribosylation, this time a trend could be seen in all three membrane fractions. This trend was observed in each membrane fraction both at low drug concentrations, where the drugs were soluble, and high concentrations, where three of the drugs became insoluble. There was definitely greater inhibition of cyclase activity occurring with chlorpromazine, trifluoperazine and triflupromazine than with the other three drugs (Fig. 5-4A, B and C). The order for greatest inhibition appears to be trifluoperazine > chlorpromazine, triflupromazine > amitriptyline > promazine > promethazine. This correlates very well with the known IC_{50} values (Fig. 4-1) for the inhibition of calmodulin activity (Prozialeck and Weiss, 1982), as well as with the known antidiarrhoeal activities of the drugs (Weiss *et al.*, 1982; Zavecz *et al.*, 1982); chlorpromazine, trifluoperazine and triflupromazine have greater antidiarrhoeal activities than the other drugs.

Therefore, the results of this experiment do show that there is a relationship between the binding of the drugs to calmodulin, with their effect on the movement of ions across the brush border membrane and with their effect on adenylate cyclase activity.

CHAPTER SIX

PHOSPHORYLATION

6.1 Introduction

The cyclic AMP resulting from the activation of adenylate cyclase by cholera toxin in the basal-lateral membrane (Chapter Five), which resulted from the activation of the α-subunit of the regulatory component of adenylate cyclase ($G_{s\alpha}$) at the brush border membrane (Chapter Four), exerts its effect by activating intestinal cyclic-AMP-dependent protein kinases (Alhanaty and Shaltiel, 1979) at the brush border membrane. It is thought that the phosphorylation and dephosphorylation of brush border membrane proteins is responsible for controlling the movement of electrolytes across the small intestinal epithelium (Scalera *et al.*, 1983); the major secretory response occurs in the brush border membrane (Frizzell *et al.*, 1979). The action of cholera toxin, mediated through cyclic AMP, may result in an increase in the phosphorylation of certain brush border membrane proteins and/or may cause the phosphorylation of unique membrane proteins. This may be responsible for the decrease in Na^+-Cl^- absorption and increase in Cl^- secretion, which results in the passive movement of water from the cell, producing the characteristic diarrhoea of the disease. Indeed, evidence has been provided showing the activation of a Cl^- channel by the cyclic-AMP-dependent phosphorylation of one or more membrane proteins (van Dommelen and de Jonge, 1984) and a protein of 86 kDa has been found to be phosphorylated by cyclic AMP in rat intestinal brush border membranes (de Jonge, 1976; Scalera *et al.*, 1983). In this study the pattern of phosphorylation in the brush border, basal-lateral and mixed plasma membrane

fractions was studied and compared in the presence of the toxin. The cholera-toxin-induced labelling pattern of brush border membrane proteins was compared to the pattern produced in the absence of the toxin to identify any newly labelled proteins and any increase in the labelling of proteins that were phosphorylated in the absence of the toxin.

Unfortunately, due to a lack of time, it was not possible to investigate the effect of the antipsychotic phenothiazine drugs (Fig. 4-1) on phosphorylation, but as with adenylate cyclase (section 5.4), there was good reason to believe that they would have an effect on the phosphorylation of membrane proteins. This was because the drugs bind calmodulin in a Ca^{2+}-dependent manner with high affinity and specificity (Prozialeck and Weiss, 1982), which would decrease the binding of the Ca^{2+}-activated calmodulin to protein kinases, thus reducing the activity of the kinases as well as the phosphorylation of proteins, and so alleviating the defect in the movement of ions across the membrane; the binding of Ca^{2+}-activated calmodulin activates protein kinases (Srivastava *et al.*, 1979; Kennedy and Greengard, 1981).

6.2 The cholera toxin induced phosphorylation of membrane proteins

6.2.1 Method

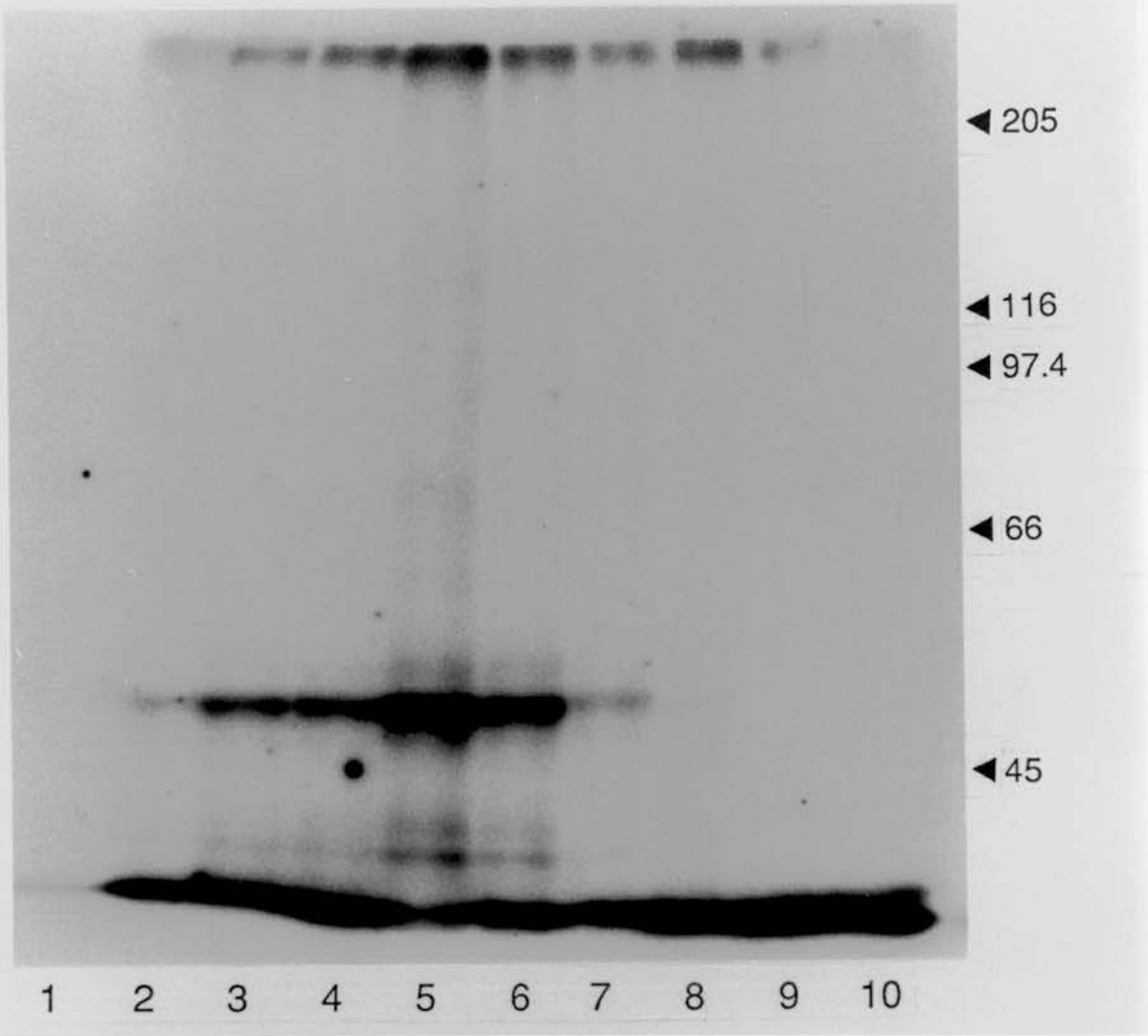
Membranes were incubated with 20 μg/ml preactivated cholera toxin and 10 μM-[γ-^{32}P]ATP in a reaction volume of 0.1 ml at 30°C for various times, as outlined in section 2.2.4.

6.2.2 Results and discussion

Initially, brush border membranes were incubated as above for times of 0-60 min to see what kind of phosphorylation pattern was obtained. The resulting autoradiographic profile (Fig. 6-1) showed one major labelled band, corresponding to a molecular mass of 53 kDa on the 10% polyacrylamide gel. The incorporation of label on this protein increased up to about 2 min; after this time the protein was dephosphorylated, and by about 20 min hardly any labelling was observed. There were other weaker labelled bands corresponding to molecular masses of 45 and 42 kDa (Fig. 6-1), and the labelling of these proteins also reached a maximum at about 2 min. However, the proteins ran much faster than usual for a 10% polyacrylamide gel, and so some of the lower molecular mass proteins might have either run with the dye front or have run off the gel completely. Since there was obviously something wrong with the gel it could not be determined whether or not there were any lower molecular mass proteins that were labelled. Therefore, fresh solutions for making the gels were made and the experiment was repeated both in the presence and absence of cholera toxin for times of 20 s to 10 min. This would show

Fig. 6-1 Incorporation of ^{32}P-phosphate into brush border membrane proteins

Brush border membranes were incubated with 20 μg/ml preactivated cholera toxin and 10 μM-[γ-^{32}P]ATP for times of 0 min (lane 1), 20 s (lane 2), 40 s (lane 3), 1 min (lane 4), 2 min (lane 5), 5 min (lane 6), 10 min (lane 7), 20 min (lane 8), 40 min (lane 9) and 60 min (lane 10) at 30°C. Membranes (50 μg membrane protein per lane) were run on a 10% polyacrylamide gel and the resulting autoradiograph is shown above. Migration of molecular mass standards expressed in kDa is as indicated.


205
116
97.4
66
45
1
2
3
4
5
6
7
8
9
10

any difference in labelling as a result of adding the toxin. The results of the experiment (Fig. 6-2) show that as well as the 53, 45 and 42 kDa proteins, two other proteins were also labelled; one of 36 kDa, which was phosphorylated after an incubation time of about 5 min, and one of 30 kDa, which was very weakly labelled and was phosphorylated after an incubation time of about 2 min. Interestingly, in the absence of cholera toxin only two proteins appeared to be labelled. These were the ones of 53 and 42 kDa, and both were phosphorylated to a much smaller extent than in the presence of the toxin. It is possible that the proteins of 45 and 30 kDa were also phosphorylated, but did not show up on the autoradiograph because they were only weakly labelled in comparison to the proteins of 53 and 42 kDa, as was the case in the presence of the toxin. However, the protein of 36 kDa was definitely not phosphorylated in the absence of the toxin, since in the presence of toxin it was labelled to a much greater extent than the 42 kDa protein and yet the labelling of this 42 kDa protein showed up in the absence of toxin.

The next stage was to look at the phosphorylation of proteins in the mixed plasma and basal-lateral membrane fractions (Fig. 6-3) and compare the patterns obtained to that which was found for brush border membranes (Fig. 6-1 and Fig. 6-2). Figure 6-3 shows that essentially no labelling of basal-lateral membrane proteins was obtained (Fig. 6-3B), except a faint band corresponding to the 53 kDa protein; however there was labelling of the 53, 42 and 36

Fig. 6-2 Effect of cholera toxin on the phosphorylation of brush border membrane proteins

Brush border membranes were incubated with 10 μM-[γ-^{32}P]ATP in both the presence (A) and absence (B) of 20 μg/ml preactivated cholera toxin for times of 20 s (lane 1), 40 s (lane 2), 1 min (lane 3), 2 min (lane 4), 5 min (lane 5) and 10 min (lane 6) at 30°C. Membranes (50 μg membrane protein per lane) were run on a 10% polyacrylamide gel and the resulting autoradiograph is shown above. Migration of molecular mass standards expressed in kDa is as indicated.

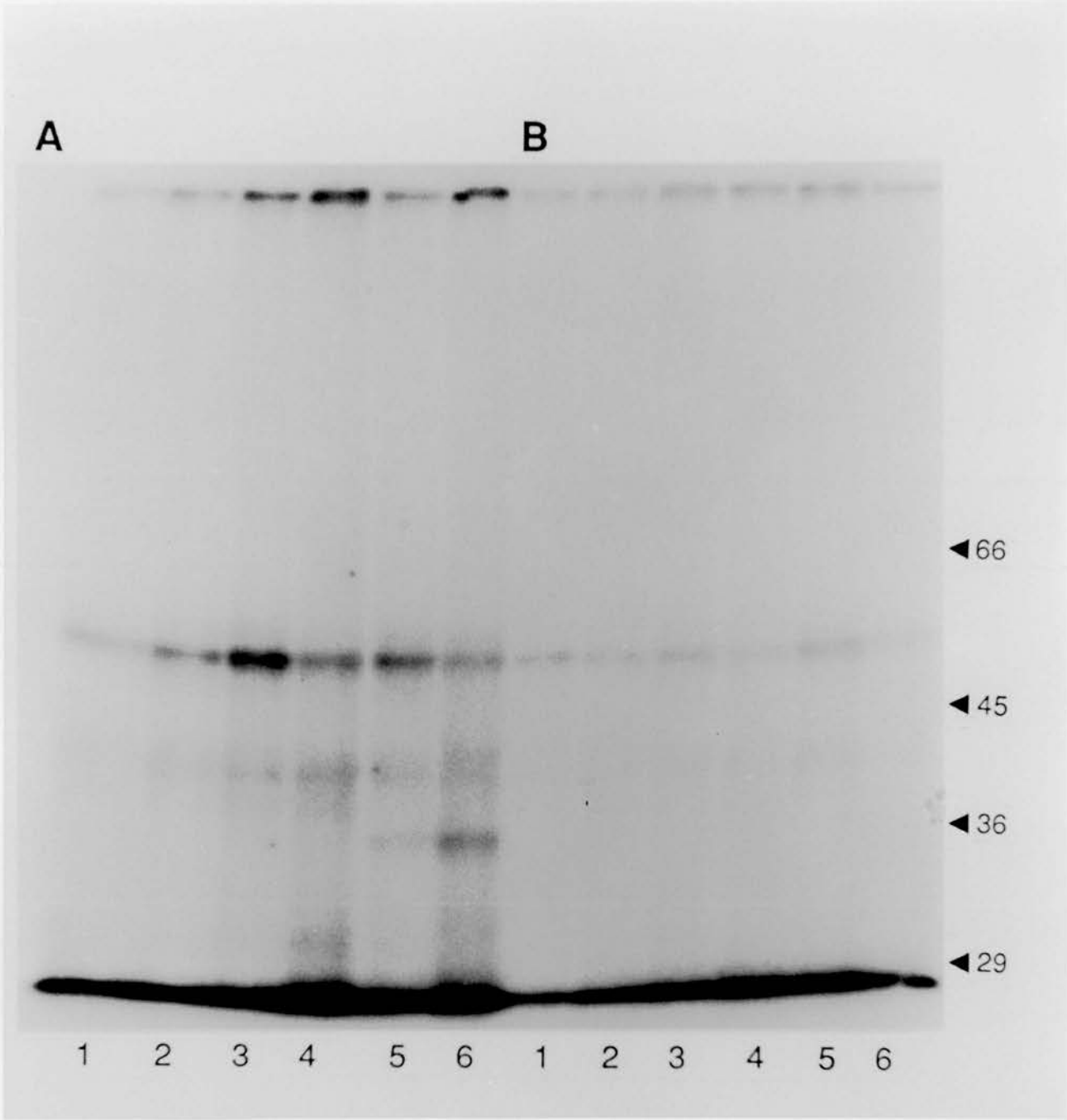
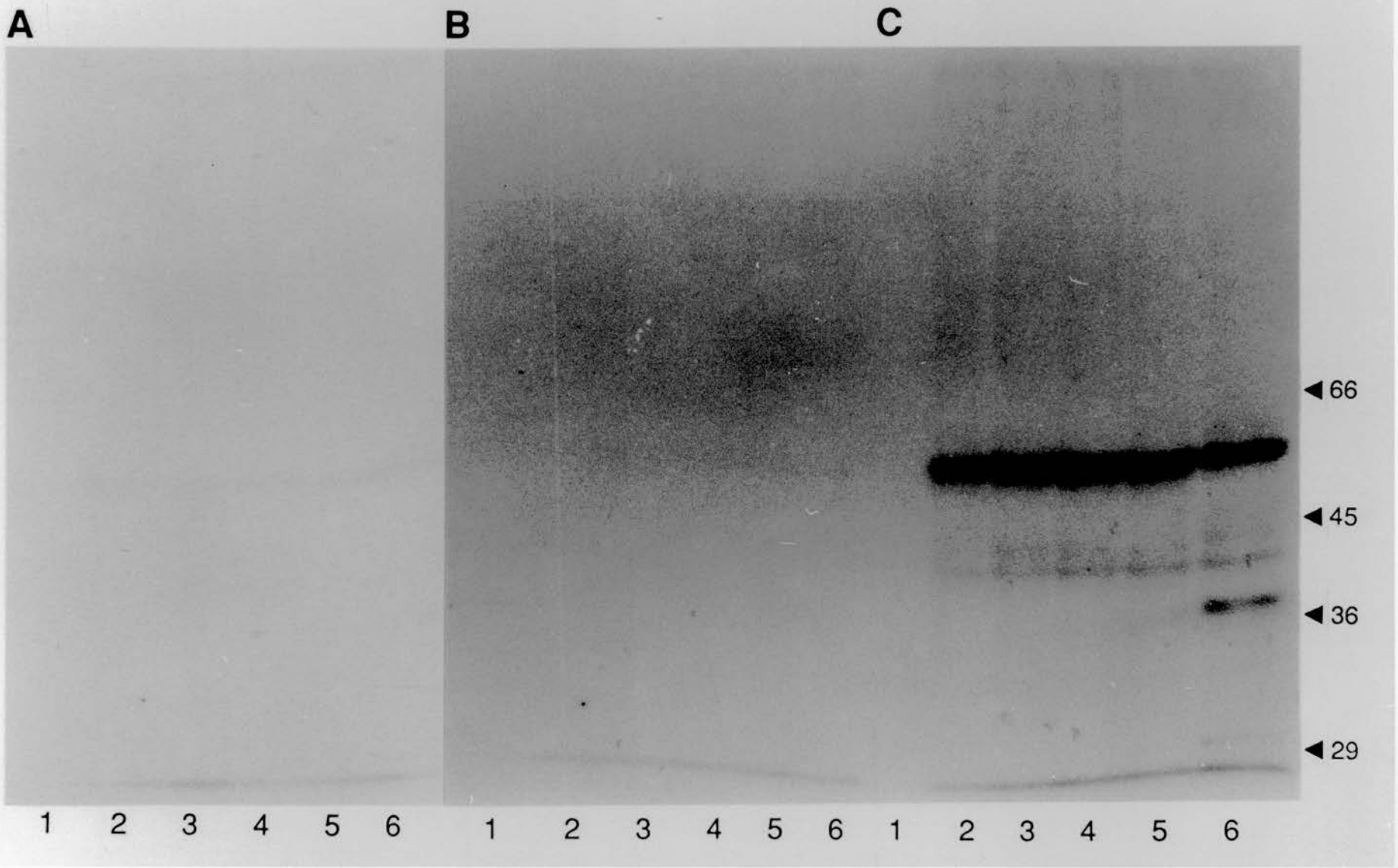

A
B
66
45
36
29
1 2 3 4 5 6 1 2 3 4 5 6

Fig. 6-3 Phosphorylation of membrane proteins

Mixed plasma membranes (A), basal-lateral membranes (B) and brush border membranes (C) were incubated with 10 μM-[γ-^{32}P]ATP and 20 μg/ml preactivated cholera toxin for times of 0 min (lane 1), 30 s (lane 2), 1 min (lane 3), 2 min (lane 4), 5 min (lane 5) and 10 min (lane 6) at 30°C. Membranes (50 μg membrane protein per lane) were run on a 10% polyacrylamide gel and the resulting autoradiograph is shown above. Migration of molecular mass standards expressed in kDa is as indicated.


A
B
C
66
45
36
29
1 2 3 4 5 6
1 2 3 4 5 6 1 2 3 4 5 6

kDa proteins in the mixed plasma membrane fraction (Fig. 6-3A), although this was also very weak. Therefore, there appear to be two brush border membrane proteins, with molecular masses of 53 and 42 kDa, whose labelling was increased in the presence of cholera toxin, and certainly one uniquely labelled protein of 36 kDa and maybe two of 45 and 30 kDa which were labelled only in the presence of the toxin. There was no labelling of a 86 kDa protein as was found with rat intestinal brush border membranes by de Jonge (1976) and Scalera *et al.* (1983). As previously mentioned, the phosphorylation of proteins in the basal-lateral membrane fraction was virtually nonexistent, but the faint labelling observed of the 53 kDa protein was probably due to crosscontamination of the fraction with brush border membranes as discussed in section 3.3.5. The assay conditions need to be optimized further to try and increase the labelling of the minor phosphorylated proteins or maybe to achieve the phosphorylation of new proteins. Oddly, since cholera toxin action results in a decrease in Na^+-Cl^- absorption and an increase in Cl^- secretion, and since the phosphorylation of brush border membrane proteins is thought to be responsible for these changes in ion movement, then because a continual loss of water is observed in the disease, any cholera-toxin-induced phosphorylation would be expected to be permanent. This, as shown in Figure 6-2, is certainly not the case, as was also found by Scalera *et al.* (1983). They observed that the *in vivo* incorporation of ^{32}P into brush border membrane proteins increased up to 60 min and remained constant afterwards, but state that *in vitro*

phosphorylation for times greater than 20 s led to considerable dephosphorylation reactions. Under the experimental conditions adopted in this study this dephosphorylation cannot be due to the level of cyclic AMP being reduced due to a depletion in NAD^+ content. It is more likely to be due to differences in the structure and organization of the membranes of *in vivo* and *in vitro* systems, affecting protein kinases and ^{32}P-acceptor sites as suggested by Scalera *et al.* (1983).

In conclusion, although the assay appears to be working, it is possible that the phosphorylation seen is not of any particular physiological interest or at least is not the phosphorylation responsible for controlling the ion transport changes occuring at the brush border membrane. However, it is clear that there are some interesting cholera toxin effects at the brush border membrane and that more work needs to be done on this phosphorylation assay, particularly before attempting to look at any drug effects.

CHAPTER SEVEN

DISCUSSION

AND

FUTURE WORK

The main purpose of this study was to investigate the mechanisms by which cholera toxin causes the massive secretion of fluid from intestinal cells, as well as to look at the effects of antidiarrhoeal drugs on these processes. Although a lot is known about the events leading to the water loss, most of the work has been done using cell types not involved with the pathology of the disease (Gill and Meren, 1978; Cassel and Pfeuffer, 1978; Northup *et al.*, 1980; Becker and Blecher, 1981; Raufman and Cosowsky, 1987), and so it seemed necessary to look specifically at effects on intestinal epithelial cells. It is hoped that eventually the results obtained from studying these mechanisms will help in improving the rational design of antidiarrhoeal drugs, and ultimately improve prophylaxis or the treatment of cholera and other diarrhoeal disease.

Initially, a mixed plasma membrane preparation, consisting of brush border and basal-lateral membranes, was prepared by differential centrifugation from rabbit intestinal epithelial cell scrapings. The brush border and basal-lateral membranes were separated by $MgCl_2$ precipitation and the purification assessed by marker enzyme assays.

Three proteins of 45, 40 and 37 kDa were found to be ADP-ribosylated by cholera toxin in brush border membranes, and possibly to a much smaller degree in basal-lateral membranes; the vast majority of the ADP-ribosylation observed in the basal-lateral membrane fraction was due to crosscontamination with brush border membranes, as judged by marker enzyme assay results. The 45 kDa protein has the

same molecular mass as the α subunit of the regulatory component of other adenylate cyclases, but the 40 kDa protein which was the major ADP-ribosylated protein may be $G_{s\alpha}$ since the molecular mass of $G_{s\alpha}$ has not been determined for intestinal cells. The identity of $G_{s\alpha}$ should be determined by immunological means initially, and then by amino acid sequencing. Any relationship between the 45, 40 and 37 kDa proteins should be investigated by purifing the proteins by running them in a second dimension (eg isoelectric focussing). They could then be digested with proteolytic enzymes, to look for any similarities between them, and they could also be at least partially sequenced. Determination of their amino acid sequences would also aid in their identification, since they could then be compared with other published protein sequences, including components of the adenylate cyclase complex that have been purified in other cells, which are stored in data-bases. The 37 kDa protein is unusual in that it does not seem to have been reported in the literature as a protein that is ADP-ribosylated. This protein, though, does have a molecular mass similar to that of the β subunit of G_s (35 kDa) (for example see Neer and Clapham, 1988), and so its amino acid sequence should be also compared to known β subunit sequences, even though the β subunit has not been reported to be ADP-ribosylated. Evidence was also provided showing that about 20% of the 45 and 40 kDa proteins are released from the brush border membrane upon ADP-ribosylation. Further experiments should be carried out to see if this release increases with time, ensuring that there is sufficient [adenylate-^{32}P]NAD$^+$ in the reaction mixture.

Cholera toxin and sodium fluoride were both found to activate adenylate cyclase via the same regulatory component (G_s), but only in basal-lateral membranes: any activation observed in the brush border membrane fraction was due to crosscontamination with basal-lateral membranes, as judged by marker enzyme assay results. The release of the $G_{s\alpha}$-like protein from the brush border membrane, where it is only to be found, explains how it can can get to the catalytic subunit of adenylate cyclase, located only in the basal-lateral membrane, to cause an increase in the production of cyclic AMP.

This increase in cyclic AMP, resulting from the activation of adenylate cyclase, caused an increase in the phosphorylation of two proteins of 53 and 42 kDa, and the unique phosphorylation of a 36 kDa protein and maybe two of 45 and 30 kDa, all in the brush border membrane. Further studies on the phosphorylation of proteins need to be done, to see if by altering conditions any new proteins can be labelled or to see if the labelling of existing proteins can be increased. Whether or not any of the above labelled proteins are physiologically relevant awaits further studies.

The results of this study agree with the following theory for the mechanism of action of cholera toxin. The active A_1 peptide of cholera toxin catalyzes the ADP-ribosylation of the α subunit of the stimulatory regulatory component of adenylate cyclase, which is located only in the brush border

membrane. This ADP-ribosylation causes the release of the α subunit from the membrane, which then moves through the cell cytosol to the basal-lateral membrane where it activates the catalytic component of adenylate cyclase. The resulting increase in the cyclic AMP content of the cell activates cyclic-AMP-dependent protein kinases causing the phosphorylation of brush border membrane proteins. This phosphorylation is thought to mediate the movement of ions across the membrane; specifically it is thought to cause a decrease in Na^+-Cl^- absorption in villus cells (Fan and Powell, 1983) and an increase in Cl^- secretion in crypt cells (van Dommelen and de Jonge, 1984).

One of the main things that still needs to be done is to investigate the effect of cholera toxin on ion transport. This would involve measuring the uptake of sodium and chloride into vesicles prepared from the membranes using radioactive markers. This procedure (for example see van Dommelen and de Jonge, 1984) is accepted as a reasonable working model of the intact epithelium, and would provide a useful tool for looking at how biochemical changes might affect secretion. A correlation between ADP-ribosylation and/or adenylate cyclase activity and variations in secretion, in vesicles made from both fractions of the purified membrane and in intact cells, could then be investigated. It could be determined whether the reappearance of phosphorylated protein bands coincided with changes in ion secretion. If such bands existed then they could be possibly identified by comparing them with those

that have already been shown in published papers (for example see de Jonge and Lohmann, 1985). The proteins could also be analyzed by digestion, sequenced and the sequences compared to those in data-bases as before. It should be remembered that it is not absolutely proved, even if always assumed, that the fluid loss is actually affected by adenylate cyclase at all: some workers favour guanylate cyclase to be responsible for the changes in ion transport at the brush border membrane (de Jonge, 1976; Scalera *et al.*, 1983). The changes in the movement of ions may also be due to a calcium effect (Bolton and Field, 1977) resulting from an activation of the phosphatidyl inositol lipid signalling pathway.

As far as the drug aspect of the project was concerned, all of the six drugs tested were found to inhibit both the ADP-ribosylation of proteins and the activation of adenylate cyclase. Specifically, the drugs inhibited adenylate cyclase activity to the greatest extent in the order trifluoperazine > chlorpromazine, triflupromazine > amitriptyline > promazine > promethazine. This correlates very well with the published antidiarrhoeal activities of the drugs (Weiss *et al.*, 1982; Zavecz *et al.*, 1982), as well as with the published IC_{50} values for the inhibition of calmodulin activity (Prozialeck and Weiss, 1982), i.e. trifluoperazine, chlorpromazine and triflupromazine have greater antidiarrhoeal activity and lower IC_{50} values than the other three drugs. This also seems to be the case for the inhibition of ADP-ribosylation, but is not as obvious.

These results are obviously only preliminary and further work needs to be done, particularly because of the drug solubility problem, but they do show that the antipsychotic phenothiazines do inhibit ADP-ribosylation and adenylate cyclase activity.

Unfortunately, the effects of the drugs on phosphorylation were not investigated because the phosphorylation assay itself has not yet been fully characterized. However, it should be stated that the phosphorylation assay cannot be used as it stands when looking at the effect of the drugs on phosphorylation. The assay was designed so that the ADP-ribosylation of G_s and the increase in production of cyclic AMP would occur simultaneously, to ensure that as soon as the cyclic AMP was produced it would activate protein kinase and cause the phosphorylation of membrane proteins. This means that the effects of drugs on the phosphorylation pattern would be difficult to interpret, since it would be impossible to tell whether any reduction in the phosphorylation of proteins was due to the inhibition of protein kinase activity, or whether it was due to the inhibition of adenylate cyclase activity as well as the inhibition of ADP-ribosylation. The problem with separating the phosphorylation assay from the adenylate cyclase assay and the ADP-ribosylation assay, is that unlabelled ATP would have to be added to the adenylate cyclase assay to produce cyclic AMP, and so when the adenylate cyclase reaction mixture is added to the phosphorylation assay mixture containing [γ-^{32}P]ATP there would be no incorporation of

label, since the proteins would have been already phosphorylated with the unlabelled ATP. The problem, therefore, lies in the fact that both adenylate cyclase and protein kinase require ATP for producing cyclic AMP and phosphorylating proteins respectively, and because protein kinase requires the cyclic AMP produced by adenylate cyclase for its activity, thus making it impossible to separate the two processes so that the phosphorylation can be studied independently. The answer may be that the assays need not be separated at all, it may be simply a question of there being a difference in the times taken to inhibit the different processes, particularly since phosphorylation occurs so rapidly: the time taken to inhibit ADP-ribosylation and adenylate cyclase activity for a particular drug concentration may not be as fast as the time taken to inhibit protein kinase activity. However, once the effects of the drugs on the phosphorylation of membrane proteins are known, their effects on ion flux should be studied *in vitro* and, if possible, *in vivo* to see if the drugs inhibit the secretory and/or the antiabsorptive effects of the toxin. If the drugs inhibit the cholera-toxin-induced phosphorylation of different proteins, and also inhibit different ion transport processes, then the results may give some clues as to the function of the proteins.

Finally, changes in the calcium and calmodulin concentrations should be looked at more directly, and other second messenger systems, such as cyclic GMP and inositol phosphate and its derivatives, should be investigated.

The results presented in this report have gone someway in helping to understand the mechanisms by which cholera toxin causes the disease cholera, but as indicated in this chapter there are still many things that need to done before any advances can be made in developing more effective drugs.

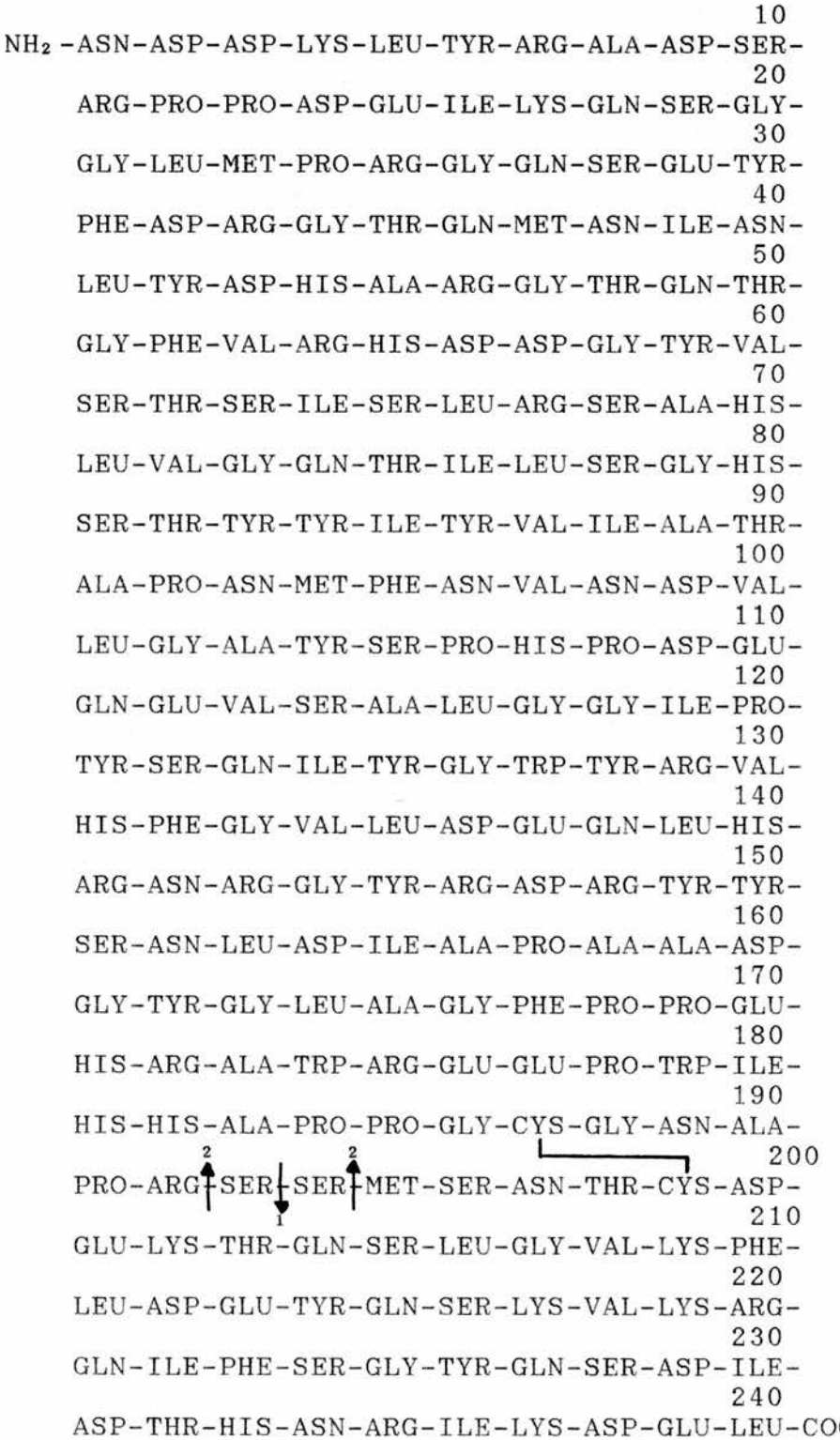
APPENDIX A The primary structure of cholera toxin B subunit

The complete amino acid sequence of the B subunit was determined from the nucleotide sequence of the single ctx operon copy of strain 2125 (El Tor biotype) (Mekalanos *et al.*, 1983).

```
                                                        10
NH2 -THR-PRO-GLN-ASN-ILE-THR-ASP-LEU-CYS-ALA-
                                                        20
     GLU-TYR-HIS-ASN-THR-GLN-ILE-TYR-THR-LEU-
                                                        30
     ASN-ASP-LYS-ILE-PHE-SER-TYR-THR-GLU-SER-
                                                        40
     LEU-ALA-GLY-LYS-ARG-GLU-MET-ALA-ILE-ILE-
                                                        50
     THR-PHE-LYS-ASN-GLY-ALA-ILE-PHE-GLN-VAL-
                                                        60
     GLU-VAL-PRO-SER-SER-GLN-HIS-ILE-ASP-SER-
                                                        70
     GLN-LYS-LYS-ALA-ILE-GLU-ARG-MET-LYS-ASP-
                                                        80
     THR-LEU-ARG-ILE-ALA-TYR-LEU-THR-GLU-ALA-
                                                        90
     LYS-VAL-GLU-LYS-LEU-CYS-VAL-TRP-ASN-ASN-
                                                       100
     LYS-THR-PRO-HIS-ALA-ILE-ALA-ALA-ILE-SER-
              103
     MET-ALA-ASN-COOH
```

APPENDIX B The primary structure of cholera toxin A subunit

The complete amino acid sequence of the A subunit was determined from the nucleotide sequence of the single ctx operon copy of strain 2125 (El Tor biotype) (Mekalanos *et al.*, 1983). The A subunit is cleaved either at position 1 or at position 2 (with the loss of two serine residues) producing the peptides A_1 (1-193) and A_2 (194-240). The disulphide bond between A_1 and A_2 is indicated.



APPENDIX C Adenylate cyclase assay: calculation of results

[based on the method of Salomon (1979)]

A = ATP (pmol per assay tube)

E = enzyme (μg membrane protein per assay tube)

Primed letters = ^{32}P channel in scintillation counter

Unprimed letters = ^{3}H channel in scintillation counter

H, H′ = cpm standard cyclic [8-^{3}H]AMP

P, P′ = cpm standard [α-^{32}P]ATP

S, S′ = cpm sample

All values have the background value subtracted

The specific radioactivity of [α-^{32}P]ATP (in cpm/pmol) with a 1:100 dilution is

$$R_{sp} = \frac{P' \times 100}{A}$$

The correction for ^{3}H counts in the ^{32}P channel is

$$F = \frac{H' \times S}{H}$$

The amount of membrane protein per assay is $\frac{E}{1000}$ mg

The recovery from the columns is $\frac{S}{H} \times 100\%$

Therefore, the gross amount of cyclic AMP produced in each assay tube (in pmol/mg protein for time t) is

$$\text{cyclic AMP}_{gross} = \frac{H}{S} \times \frac{(S' - F) \times 1000}{R_{sp} \times E}$$

The blank value (Z) is calculated similarly, and the net amount of cyclic AMP produced (in pmol/mg protein for time t) is obtained by subtracting Z from the gross value:

$$\text{cyclic AMP}_{net} = \text{cyclic AMP}_{gross} - Z$$

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